CONTROL OF DEVELOPMENT BY PHYTOCHROME IN THE GARDEN PEA (Pisum sativum L.)

by

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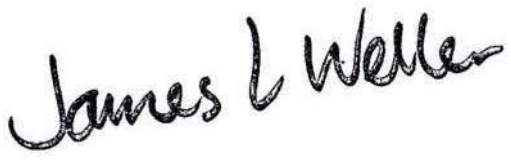
DECLARATION

This thesis does not contain any material which has been submitted for the fulfilment of any other degree or diploma. To the best of my knowledge and belief, this thesis contains no material which has been published, written, or provided by another person, except where due reference is given.



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ABSTRACT

The phytochrome family is a well-known and important group of biliprotein photoreceptors which mediate effects of light on plant development. This thesis comprises an investigation of the physiological roles of different forms of phytochrome in the garden pea (*Pisum sativum L.*). The presented work focuses on the isolation and subsequent physiological characterization of various phytochrome-deficient mutants of pea, including mutants deficient in phytochrome A, phytochrome B and phytochrome chromophore synthesis. Ethylmethanesulfonate mutagenesis was employed to induce mutation in the pea cultivar Torsdag. M_2 seedlings were screened under red (R) or far-red light (FR) conditions to identify potential phytochrome-deficient mutants. All candidate mutants were back-crossed to the parental line to establish the nature of the genetic control of the mutant phenotypes, and complementation testing was performed between mutant lines showing similar alterations to spectral sensitivity. Four distinct loci were defined by mutants isolated in these screens, corresponding to the previously described *LV* locus and three novel loci designated *FUN1*, *PCD1* and *PCD2*. *LV* and *FUN1* were mapped to linkage groups VI and II, respectively.

Apoprotein-deficient mutants. Mutant *lv* plants are deficient in a phyB apoprotein and show reduced responses to R, while *fun1* mutants are deficient in phytochrome A apoprotein and are unresponsive to FR. Physiological studies suggest that phyB controls seedling phytochrome responses in the LFR mode, whereas phyA controls responses in the VLFR and HIR modes. Double mutants lacking both phyA and phyB reveal (a) that phyA is the only phytochrome controlling responses to continuous FR (b) that both phyA and phyB are required for normal responses to continuous R, and (c) neither phyA nor phyB play a substantial role in inhibition of stem elongation by blue light. Examination of mature plant photoresponses revealed a continuing role for both phyA and phyB in the maintenance of full de-etiolation, and an important role for phyA in photoperiod detection and flower induction.

Chromophore-deficient mutants. Mutant *pcd1* and *pcd2* plants show a reduction in both phyA- and phyB-mediated responses at the seedling stage and a severe depletion of spectrally active phytochrome, consistent with deficiency in the common tetrapyrrole chromophore, phytochromobilin (PΦB). *In vitro* assembly studies, *in vivo* feeding of PΦB precursors, and HPLC-based assay of heme and biliverdin (BV) IXα metabolism in isolated plastids revealed the *pcd1* and *pcd2* mutants to be deficient in the conversion of heme to BV IXα and BV IXα to PΦB, respectively.

Other mutants. In addition to the mutants described above, a dominant mutant (AF05) with strongly enhanced responses to light was also isolated. This mutant has a seedling and flowering phenotype similar to phyA-overexpressing transgenic lines in other species. In addition, the AF05 mutation maps close to *FUN1*, suggesting possible allelism between these loci.

Substantial portions of this thesis have already been published, as follows;

Weller JL, Reid JB (1993) Photoperiodism and photocontrol of stem elongation in two photomorphogenic mutants of *Pisum sativum* L. Planta 189, 15-23

Weller JL, Murfet IC (1994) Location of the *Lv* gene in linkage group VI. Pisum Genet. 26, 41-43

Weller JL, Nagatani A, Kendrick RE, Murfet IC, Reid JB (1995) New *lv* mutants of pea are deficient in phytochrome B. Plant Physiol. 108, 525-532

Weller JL, Terry MJ, Rameau C, Reid JB, Kendrick RE (1996) The phytochrome-deficient *pcd1* mutant of pea is unable to convert heme to biliverdin IXα. Plant Cell 8, 55-67

These papers are included at the end of the thesis, together with other papers published during the course of the work.

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ABBREVIATIONS

Abbreviations are defined at first appearance in the text. Standard phytochrome terminology and abbreviations are employed (Quail et al. 1994). For convenience all non-standard abbreviations used are listed below.

a a	amino acid
ALA	5-aminolevulinic acid
B	blue (light)
BV	biliverdin
Chl	chlorophyll
cv.	cultivar
d	day
EMS	ethylmethanesulphonate
EOD	end-of-day
FLR	flower/leaf relativity
FR	far-red (light)
FT	flowering time
G	green (light)
h	hour
HIR	high-irradiance response
IL	white light from incandescent globes
LA	leaflet area
LD	long day(s)
LDP	long-day plant
LE	leaves expanded
LFR	low-fluence response
Lx-y	length between nodes x and y
NFD	node of first open flower
NFI	node of flower initiation
PAL	cv. Paloma
PAR	photosynthetically active radiation
PCB	phycocyanobilin
PFB	phytochromobilin
R	red (light)
RN	reproductive nodes
SD	short day(s)
SDP	short-day plant
SOL	cv. Solara
TBST	Tris-buffered saline/Tween
TN	total nodes
TOR	cv. Torsdag
VLFR	very-low-fluence response
WFL	light from cool white fluorescent tubes
WL	white light
WT	wild type

1. Photomorphogenesis in Higher Plants

1.1. Introduction

Whereas animals can exert control over energy availability by altering their location, most plants are not able to do this. Instead, they employ a strategy in which they control their energy intake by altering their basic morphology and developmental strategy in response to environmental signals. Since plants derive virtually all of their energy from light, it is not surprising that light is one of the most important such signals. The incident light encountered by a plant may vary with respect to spectral quality, intensity, direction and duration, and plants are capable of detecting variation in each aspect. Light thus presents a relatively complex signal, to which plants respond in a variety of ways throughout their life cycle. Light can influence germination, leaf and chloroplast development, floral induction and senescence, and also affects plant architecture by inhibiting stem elongation and reducing apical dominance. These influences collectively constitute a developmental program referred to as photomorphogenesis. Two other important modes of developmental response to light are often distinguished from photomorphogenesis; photoperiodism, in which plants respond to variations in the daily cycle of light and dark, and phototropism, in which growing apices reorient according to directional asymmetry in the incident light.

Some of the most important aspects of photomorphogenesis are best illustrated in a comparison of seedlings grown in darkness and in continuous white light (**Figure 1.1A**). Seedlings grown in darkness consist almost entirely of stem tissue, which undergoes very rapid elongation. Leaves are present but expand and develop only minimally, and the stem immediately below the apical bud is bent to form a hook. Dark-grown or etiolated plants thus display a "light-seeking" developmental strategy in which resources are strongly directed toward stem elongation and away from development of photosynthetic organs. This developmental program has also been referred to as skotomorphogenesis. In contrast, plants grown in white light show maximal direction of resources towards the development of photosynthetic tissue at the expense of stem tissue, and the establishment of a robust growth habit (a "light-utilising"strategy). The complex transition from a light-seeking to a light-utilising strategy upon exposure to light is referred to as de-etiolation.

While in most plants full de-etiolation requires long exposures to white light (WL) at high intensity, partial de-etiolation may be induced by exposure to light in more restricted regions of the spectrum. Action spectra constructed for various developmental

responses identified the blue (B; 400-450 nm), red (R; 640-680 nm) and far-red (FR; 710-750 nm) regions of the spectrum as regions of particular importance (e.g. Flint 1936, Went 1941, Parker et al. 1949). An indication of the relative importance of these various regions in early development is illustrated by growing seedlings in continuous monochromatic light. (**Figure 1.1B**). In pea, both red and blue light are relatively effective for the inhibition of stem elongation, whereas FR at a similar fluence rate is somewhat less effective.

These differential effects suggest the operation of several different photoreceptor systems, and research in photomorphogenesis has long been directed toward identification of the photoreceptors responsible for mediation of responses to light in these wavebands (Sage 1992, Galland 1992). Responses to the R and FR regions of the spectrum are now known to be largely mediated by a group of biliprotein photoreceptors known as the phytochromes (Quail et al. 1995, Smith 1995, Furuya and Schäfer 1996), whereas responses to B are at least in part mediated by flavoprotein photoreceptors known as cryptochromes (Ahmad and Cashmore 1996). Rapid progress in recent years has stemmed mainly from the characterisation of photoreceptors at the molecular level, combined with identification and physiological study of mutants defective in specific photomorphogenic responses. Most recent work has concentrated on *Arabidopsis thaliana* (L.) Heynh. as a model system, in view of its unquestionable advantage for both classical and molecular genetics (Quail et al. 1995). To a lesser extent, tomato (*Lycopersicon esculentum* Mill.) has also been used as a model system, again, with considerable success (Kendrick et al. 1994). However, there is a continuing need for the development of additional model species representing as wide a range as possible of the great variety in plant growth habit and developmental strategy.

As an introduction to the experimental work presented in the body of this thesis, this chapter provides an overview of current understanding of the structure and function of the photoreceptors controlling photomorphogenesis in higher plants. This topic has attracted intense interest over the last decade, and has been the subject of numerous reviews (e.g. Furuya 1989, Smith and Whitelam 1990, Tomizawa et al. 1990, Chory 1991, Kendrick and Nagatani 1991, Quail 1991, Galland 1992, Reed et al. 1992, Chory 1993, Furuya 1993, Kaufman 1993, Terry et al. 1993b, Vierstra 1993, Elich and Chory 1994, Deng 1994, Kendrick and Kronenberg 1994, Liscum and Hangarter 1994, Quail 1994, Short and Briggs 1994, Whitelam and Harberd 1994, Pratt 1995, Quail et al. 1995, Smith 1995, Ahmad and Cashmore 1996, Furuya and Schäfer 1996).

1.2. Phytochrome

The existence of phytochrome was first postulated in explanation of observations that various responses induced by R, such as seed germination and promotion of flowering in short day plants (SDP), could be reversed by subsequent FR (Borthwick et al. 1952a, b). It was subsequently demonstrated that this property of photoreversibility resided within a single pigment (Butler et al. 1959) with two forms, P_r and P_{fr}, whose absorbtion spectra (**Figure 1.2**) corresponded to action spectra for R-induction and FR-reversal of various physiological responses (Withrow et al. 1957). These findings provided a convenient *in vitro* assay for phytochrome, which enabled further purification of the pigment (Siegelman and Hendricks 1965), and the production of antisera (Pratt and Coleman 1971), culminating in immunopurification of the photoreceptor in its native form (Vierstra and Quail 1983).

The fact that many R-induced responses were found to correlate in extent with the level of spectrophotometrically detectable P_{fr} (e.g. Klein et al. 1967, Pjon and Furuya 1968) encouraged interpretation of physiological data in terms of a single pool of phytochrome. However, this relationship was found not to hold in all cases (e.g. Hillman 1965, Briggs and Chon 1966), and was challenged by the emerging distinction of diverse physiological modes of response to light in the R and FR regions of the spectrum (see below). These observations led to the suggestion that multiple functionally distinct pools of phytochrome might exist (Siegelman and Butler 1965, Hillman 1967). A variety of other spectrophotometric (e.g. Brockmann and Schäfer 1982) and physiological studies (e.g. Saji et al. 1982) supported this suggestion, which was later logically extended to consider the possible existence of multiple expressed phytochrome genes (Quail et al. 1983).

1.2.1. The Phytochrome Gene Family

Early evidence for multiple structurally distinct phytochromes came from findings that phytochrome preparations from green and etiolated plants were spectrophotometrically and/or immunochemically distinct (Tokuhisa et al. 1985, Abe et al. 1985, Shimazaki and Pratt 1985). Furthermore, the immunochemically distinct phytochromes isolated from pea (Abe et al. 1985) were also shown to differ in primary structure (Abe et al. 1989). Nucleotide sequences for distinct phytochrome-encoding genes were first obtained by Sharrock and Quail (1989) for three genes in *Arabidopsis thaliana* (*PHYA-C*). The deduced polypeptide sequences for these phytochromes share approximately 50% identity (Sharrock and Quail 1989). The complete complement of phytochrome genes in *Arabidopsis* was subsequently defined by Clack et al. (1995) who characterised two

further genes, *PHYD* and *PHYE*. Sequence comparisons showed that *PHYD* is much more closely related to *PHYB* (80%) than to *PHYA* or *PHYC* (50%). *PHYE* is also most similar to *PHYB* but shares a much lower sequence identity (56%). These results showed the phytochrome gene family in *Arabidopsis* to consist of four sub-families; A, B(D), C and E. Genetic distance analysis suggested that the divergence of *PHYA, B* and *C* occurred before or very early in angiosperm evolution, while the *PHYB/E* divergence occurred somewhat later, and the *PHYB/D* divergence took place in relatively recent evolutionary history.

More recent evidence indicates that diversification of the phytochrome gene family might be greater than previously anticipated. A survey of phytochrome sequences across a large range of phylogenetically diverse angiosperm species (Mathews et al. 1995) confirmed the hypothesis that the *PHYA, B* and *C* subgroups are relatively ancient, but also found that duplication and divergence within the phytochrome subfamilies appears in many cases to have occurred recently and to a varying extent across the species examined. Thus, although several species were found to have two or more closely related genes encoding B-type (and in some cases A-type) phytochromes, these duplications appear to have occurred after the divergence of the taxa in question, indicating that orthology between B-type phytochromes from different species cannot be assumed. In addition, Mathews et al. (1995) obtained no evidence for the presence of *PHYE*-type phytochrome genes in monocotyledonous plants, suggesting that this phytochrome may be restricted to dicotyledonous species.

In the only other detailed study of the phytochrome gene family in a higher plant species, Hauser et al. (1996) obtained evidence from tomato for at least 9 phytochrome-related genomic sequences, of which at least five are expressed. These included genes corresponding to Arabidopsis *PHYA* and *PHYE*, two members of the *PHYB*-subfamily and a gene representing a new subfamily, designated *PHYF* (Hauser et al. 1996). Evidence for a *PHYC* ortholog was also obtained. Although *PHYF* has only been identified in tomato, it appears to have arisen as a relatively ancient divergence from the *PHYC* subgroup and might therefore be expected to be present in other species. The proposed evolutionary relationship between the various phytochrome genes inferred from these studies is summarised in **Figure 1.3.**

1.2.2. Phytochrome Gene Structure and Expression

All higher plant phytochrome genes examined to date encode proteins of approximately 1100 aa, with a relatively well-conserved structure. The major features of a generalised dicot *PHYA* gene and encoded protein, are illustrated in **Figure 1.4.** The phytochrome

protein is a globular, cytosolic protein consisting essentially of two domains; an N-terminal domain containing the chromophore binding site (Lagarias and Rapoport 1980), and a C-terminal domain with limited homology to bacterial sensor protein kinases (Schneider-Poetsch and Braun 1991, Thümmler et al. 1995). The number and sites of intron insertions are similar, with *PHYA*, *B* and *E* each containing three introns (Quail 1994, Clack et al. 1994), and PHYC containing only two (Cowl et al. 1994). The N-terminal domain is encoded by a single exon, whereas the C-terminal domain is encoded by the remaining smaller exons. All *PHYA* genes characterised show an additional intron in the 5' untranslated region (Quail 1994). Other notable features of phytochrome genes include the presence in *PHYB*-type genes of additional sequence coding for short C-and N-terminal extensions (Quail 1994), the presence of small open reading frames upstream of the main coding sequence in all five *Arabidopsis* genes (Sharrock and Quail 1989, Clack et al. 1994), and multiple transcription start sites in both *Arabidopsis* and pea *PHYA* genes (Sato 1988, Dehesh et al. 1994).

Sequence comparison across the five *Arabidopsis* genes identified regions of substantial conservation corresponding approximately to aa 200-460 and 520-800 in the phyA protein (Clack et al. 1994). Some indication of the function of these domains has been provided by investigation of the effects of substitution mutations and transgenic expression of *PHYA* deletion derivatives (Cherry and Vierstra 1994, Quail et al. 1995). These studies have identified regions necessary for chromophore attachment (Cherry et al. 1993), spectral integrity (Cherry et al. 1992) and dimerisation (Edgerton and Jones 1992, Cherry et al. 1993) (**Figure 1.4**). The section fromaa 520-800 includes a region in which a cluster of regulatory mutations in both *PHYA* (Xu et al. 1995) and *PHYB* (Wagner and Quail 1995) are localised. In contrast, the N-terminal 200 aa and the C-terminal 300 aa show a relatively low degree of conservation. These parts of the molecule contain regions which are necessary for the spectral integrity and full functional activity of phyA (Cherry et al. 1992, Jordan et al. 1995), and may also include determinants of its photosensory specificity (Boylan et al. 1994).

Etiolated plants have up to 100-fold more phytochrome than light-grown plants. This difference predominantly reflects the level of phyA, and is due both to down-regulation of *PHYA* expression (Quail 1994) and to destruction of phyA on conversion to P_{fr} (Vierstra 1994). Light may also influence the level of other phytochromes, but in a manner much more subtle than for phyA (Stewart et al. 1992, Wang et al. 1993). Analysis of *PHYA* promoter sequences from oat and rice have identified distinct elements necessary for the observed photoregulation of *PHYA* expression (Bruce and Quail 1990, Dehesh et al. 1990, Bruce et al. 1991). These include positive elements necessary for a high level of expression

in darkness, and a negative element, required for light-dependent repression of expression, which is also present in dicot *PHYA* genes (Dehesh et al. 1994, Quail 1994).

1.2.3. Phytochrome Chromophore Synthesis

Similarity between the action spectra for many R-induced photomorphogenic responses and the absorbance spectrum of C-phycocyanin, an accessory pigment for photosynthesis in rhodophytes and cyanobacteria, was originally noted by Parker et al. (1950). Isolation and chemical characterisation of small amounts of the phytochrome chromophore supported its identity as a linear tetrapyrrole (Siegelman et al. 1966). This was confirmed by Rüdiger and Correll (1969). Subsequent studies lead to determination of the complete structure of the chromophore, phytochromobilin (**Figure 1.5A**), and demonstration of its linkage to the phytochrome apoprotein (Rüdiger et al. 1980, Lagarias and Rapoport 1980).

As a linear tetrapyrrole, it has been assumed that phytochromobilin (PΦB) shares a common synthetic pathway with other tetrapyrroles such as chlorophyll and the phycobilins. This was supported by observations that compounds which impose an early block to tetrapyrrole biosynthesis inhibit the synthesis of spectrally active phytochrome but do not alter expression of the phytochrome apoprotein (Jones et al. 1986, Konomi and Furuya 1986). Inhibitors of tetrapyrrole synthesis were subsequently used to provide a chromophore-deficient background against which putative intermediates in phytochromobilin synthesis were assessed for their ability to support chromophore synthesis. These studies demonstrated that labelled 5-aminolevulinic acid (ALA) (Elich and Lagarias 1987) and biliverdin (BV) (Elich et al. 1989) were incorporated into the phytochrome holoprotein.

Figure 1.5B shows the proposed pathway for synthesis of the phytochrome chromophore phytochromobilin (PΦB). Relatively little is known about the specific synthetic steps for BV in higher plants, but it is assumed that the pathway is similar to that in other organisms. Ferrochelatase has recently been cloned (Smith et al. 1994) and it has recently been shown that BV can be enzymatically reduced to PΦB, which can assemble with phytochrome apoprotein to give a native absorption spectrum (Terry et al. 1995). This reduction differs from the reduction of BV in mammals (to bilirubin) and phycocyanin-containing algae (to phycoerythrobilin).

Mutants deficient in synthesis of the phytochrome chromophore have been isolated in *Arabidopsis*. The *hy1*, *hy2* and *hy6* mutants have a pale, yellow-green phenotype, are less responsive than WT to continuous R and FR, and are deficient in

spectrophotometrically detectable phytochrome, but have normal phytochrome apoprotein content (Koornneef et al. 1980, Chory et al. 1989a, Parks and Quail 1989). Rescue of these mutants by growth on BV-containing media has indicated that they affect synthetic steps prior to BV formation (Parks and Quail 1991), but no further characterisation of these mutants has been reported to date. Mutants in tomato and *Nicotiana plumbaginifolia* with similar phenotypes have also been identified (Koornneef et al. 1985, Kraepiel et al. 1994).

1.2.4. Phytochrome Function

Prior to the cloning of multiple phytochrome genes, the presence of multiple functionally distinct forms of the photoreceptor could only be inferred from the existence of several different physiological response modes. Various response modes could be distinguished on the basis of fluence or fluence rate threshold, R/FR reversibility, and adherence to the Bunsen-Roscoe law of reciprocity. This law stipulates that the magnitude of a response should depend on fluence and not fluence rate (Mancinelli 1994). These response modes include include low fluence responses (LFR), very low fluence responses (VLFR) and high irradiance responses (HIR). LFRs are classical phytochrome responses induced by R, which require 1-1000 μmol m^{-2} for saturation, are reversible by FR, and show reciprocity. HIRs require long exposures to light at higher fluence rates, are dependent on wavelength, irradiance and duration of exposure, are not FR reversible, and do not show reciprocity. VLFRs are induced by fluences from 10^{-6}-10^{-3} μmol m^{-2} sec^{-1} and are not FR-reversible. (Mancinelli 1994).

Early evidence for the involvement of at least two phytochromes in the control of these responses was provided by a comparison of the *aurea* (*au*) mutant of tomato and the *long-hypocotyl* (*lh*) mutant of cucumber, in which these response modes were differentially impaired. De-etiolated *au* mutant plants retained relatively normal phytochrome responses despite a strong deficiency in spectrophotometrically-detectable phytochrome in etiolated seedlings (Adamse et al. 1988b, López-Juez et al. 1990b, Whitelam and Smith 1991). In contrast, these responses were absent in de-etiolated plants of the *lh* mutant, which had normal levels of spectrophotometrically-detectable phytochrome if grown in the dark (Adamse et al. 1987, 1988a, López-Juez et al. 1990a, Whitelam and Smith 1991). In addition, *au* seedlings were less sensitive to R and FR, whereas *lh* seedlings responded normally to continuous FR. These and other observations linked the seedling response to continuous FR (a HIR) with the presence of spectrophotometrically-detectable phytochrome, and also suggested a distinct association between LFR-type responses in seedlings and shade-avoidance responses in de-etiolated plants.

More recently, the thorough molecular characterisation of the *Arabidopsis* phytochrome gene family by Sharrock and Quail (1989) and Clack et al. (1995) has made possible a systematic analysis of the roles of each member, through the identification of mutants deficient in synthesis of specific phytochromes, and the construction of transgenic lines expressing phytochrome genes or gene derivatives. In particular, the roles of phytochromes B and A have been explored in considerable detail.

Phytochrome B

Mutants deficient in phytochrome B are now known in *Arabidopsis* (*hy3*; Somers et al. 1991, Nagatani et al. 1991), *Brassica* (*ein*; Devlin et al. 1992), cucumber (*lh*; López-Juez et al. 1992) and sorghum (ma_3^R; Childs et al. 1992). While mutants in all four species share a similar phenotypic syndrome (Smith 1995), the *Arabidopsis* mutants are by far the best characterised, and have been shown to lie within the *PHYB* gene (Reed et al. 1993). PhyB-deficient *Arabidopsis* seedlings show reduced sensitivity to continuous R and WL but respond normally to continuous FR (Koornneef et al. 1980, Somers et al. 1991, Liscum and Hangarter 1993b, Reed et al. 1994). Similarly, phyB-overexpressing seedlings show increased sensitivity to continuous R and W, but not to FR (Wagner et al. 1991, Mc Cormac et al. 1993, Wester et al. 1994). PhyB controls responses in the classical LFR mode; i.e. those which can be induced by brief saturating pulses of R and are reversible by a subsequent FR exposure. These include the promotion of seed germination (Shinomura et al. 1994) and de-etiolation (Adamse et al. 1987, Devlin et al. 1992, McCormac et al. 1993, Reed et al. 1994), as well as similar responses to end-of-day (EOD)-FR and R:FR in de-etiolated plants (Whitelam and Smith 1991, Nagatani et al. 1991, Devlin et al. 1992). In the absence of phyB, mature plants retain a somewhat etiolated appearance, with paler foliage and elongated stems and petioles (Reed et al. 1993), indicating that phyB actively promotes de-etiolation throughout the life of the plant. In addition, phyB-deficiency causes early flowering in both a long-day plant (LDP) *Arabidopsis* (Goto et al. 1991, Halliday et al. 1994, Bagnall et al. 1995) and the SDP *Sorghum* (Pao and Morgan 1986, Childs et al. 1992), indicating that phyB acts to inhibit flowering in plants of both photoperiod response types.

Most of these responses are consistent with a model for phyB action in which $P_{fr}B$ is the active form and activity of the photoreceptor depends on the proportion of $P_{fr}B$ established by the incident light. However, more detailed examination of the phenotype of phyB-altered plants with respect to such responses as the R-induced loss of gravitropism (Liscum and Hangarter 1993a, Robson and Smith 1996) and shade-avoidance

response to R:FR (McCormac et al. 1993), have led to the emerging view that P_r and P_{fr} forms of phyB may act antagonistically (Smith 1995).

The recently reported *temporarily red-light insensitive* (*tri*) mutants of tomato provide a contrast to the other phyB-deficient mutants described above. The *tri* mutants are also specifically insensitive to R, but unlike the other mutants, this insensitivity is restricted to the first two days following germination, after which *tri* seedlings regain normal sensitivity to R (Van Tuinen et al. 1995b). De-etiolated *tri* mutant plants also retain normal responses to EOD-FR and R:FR (Van Tuinen et al. 1995b). The *tri* mutant phenotype is associated with a loss of phyB1, one of the two B-type phytochromes present in tomato. (Kerckhoffs et al. 1996). Tomato phyB1 therefore appears to have a similar function to B-type phytochromes in other species, insofar as it mediates LFR-like responses to R. However, it also obviously differs from *Arabidopsis* phyB in several important respects. It remains to be seen whether phyB2 has a role more closely analogous to that of *Arabidopsis* phyB. However, given that duplication and differentiation within the phyB family appears to have occurred after the separation of the Solanaceae and the Brassicaceae (Pratt et al. 1995), the roles of individual members of the phyB subfamily may not be directly comparable between the two groups.

Phytochrome A

Although considerable circumstantial evidence suggested a role for a light-labile phytochrome in the control of responses to continuous FR, the first clear evidence for the function of phyA was obtained from transgenic *PHYA*-overexpressing plants. Early reports (Boylan and Quail 1989, Kay et al. 1989, Keller et al. 1989, Boylan and Quail 1991) characterised *PHYA* overexpressors but gave little detailed information on spectral sensitivity or specific physiological responses. More thorough characterisation revealed that *PHYA* overexpression results in the persistence of a FR-HIR in de-etiolated plants (McCormac et al. 1991), and confers increased sensitivity to both R and FR in etiolated seedlings (McCormac et al. 1993), in contrast to the R-specific effect of *PHYB* overexpression. The specific importance of phyA for responses to FR was confirmed by the finding that phyA-deficient mutants in both *Arabidopsis* and tomato are insensitive to FR (Parks and Quail 1993, Nagatani et al. 1993, Whitelam et al. 1993, van Tuinen et al. 1995a). Although phyA-deficient *Arabidopsis* mutants show relatively normal de-etiolation responses to continuous WL and R (Parks and Quail 1993, Whitelam et al. 1993), they are less responsive to R pulses (Reed et al. 1994, Parks et al. 1996), indicating that phyA also has a role in R perception, consistent with the phenotype of phyA overexpressing lines.

While mature phyA-deficient plants have relatively normal appearance when grown under continuous WL (Parks and Quail 1993, Nagatani et al. 1993, Whitelam et al. 1993), a number of different studies indicate that phyA does contribute to the maintenance of de-etiolation in green plants (Johnson et al. 1994) and has a role in the promotion of flowering in response to day-extensions with FR-rich light or to night-breaks (Johnson et al. 1994, Reed et al. 1994).

Other phytochromes

Evidence of a physiological role for other phytochromes has come predominantly from identification of residual phytochrome responses in double mutants lacking phyA and phyB. These responses include effects of R:FR and EOD-FR on leaf expansion, radial expansion of stems, and flowering (Halliday et al. 1994, Robson et al. 1994) and the promotion of flowering by night-breaks (Reed et al. 1994).

1.2.5. Basis for Functional Differentiation Within the Phytochrome Family

Attempts to define the primary mechanism of phytochrome action have so far been inconclusive (Quail et al. 1995). However, the demonstration that the C-terminal domains of phyA and phyB are functionally interchangeable (Wagner et al. 1996a) and share an important regulatory region (Wagner and Quail 1995, Xu et al. 1995), and the fact that overexpression of certain phyB deletion derivatives interferes with endogenous phyA activity (Wagner et al. 1996b) suggests that phyA and phyB may have the same primary mechanism of action, with their photosensory specificities conferred by differences in intramolecular signal transduction, subcellular localization, and patterns of expression (Quail et al. 1995, Shinomura et al. 1996).

1.3. Blue Light Photoreceptors

1.3.1 Blue-light induced de-etiolation

Both phyA and phyB-deficient *Arabidopsis* mutants have an elongated phenotype under B, indicating that the B induction of de-etiolation is partly mediated through these phytochromes (Chory 1992, Whitelam et al. 1993). However, both of these mutants, and the chromophore-deficient *hy2* mutant, retain substantial sensitivity to B, indicating the action of at least one additional photoreceptor system under shorter wavelengths (Chory 1992, Young et al. 1992, Whitelam et al. 1993).

Among a number of *Arabidopsis* long-hypocotyl mutants isolated by Koornneef et al. (1980) one mutant, *hy4*, showed a relatively specific reduction in sensitivity to B. Additional *hy4* alleles were isolated by Ahmad and Cashmore (1993) who cloned the *HY4* gene using a T-DNA tagged mutant. The gene was found to encode a 75kDa protein consisting of a 500 aa N-terminal region with homology to a B- and UV-absorbing bacterial photolyase flavoprotein, and a 200 aa C-terminal extension with homology to tropomyosin. The purified protein, designated cryptochrome (CRY1) was shown to bind FAD (Lin et al. 1995b) and to absorb in the B, G and UV-A regions of the spectrum. CRY1 did not restore photolyase activity in photolyase-deficient *E. coli* (Ahmad and Cashmore 1996), but overexpression in transgenic tobacco conferred an exaggerated response to B and to a lesser extent, to G and UV-A (Lin et al. 1995a), consistent with the absorbtion properties of the purified protein.

These results have established CRY1 as an important photoreceptor involved in the inhibition of hypocotyl elongation (Koornneef et al. 1980, Ahmad and Cashmore 1993), promotion of cotyledon opening (Liscum and Hangarter 1992), and stimulation of anthocyanin accumulation (Ahmad et al. 1996). The observed action and absorbtion spectra of CRY1 may not be entirely explained by the presence of a single FAD chromophore (Ahmad and Cashmore 1996), and some evidence suggests that the photoreceptor may also incorporate a pterin chromophore (Malhotra et al. 1995). Evidence also exists for the presence of additional *CRY1*- and photolyase-related genes in *Arabidopsis*, although the functions of these genes have yet to be determined (Ahmad and Cashmore 1996).

1.3.2. Phototropism

Phototropism is another important plant response which is known to be stimulated by blue light. *Arabidopsis* mutants with aberrant phototropic responses but normal B-inhibition of elongation have been isolated (Khurana and Poff 1989, Khurana et al. 1989, Liscum and Briggs 1995), and allocated to four complementation groups, designated *nph1*-*nph4* (Liscum and Briggs 1995). The construction of double mutants between *nph* and *hy4* mutants has demonstrated that CRY1 does not act as the photoreceptor for this response (Liscum et al. 1992, Liscum and Briggs 1995). Severe *nph1* alleles are insensitive to both B and G as well as to UV-A, indicating that the *NPH1* gene product is necessary for phototropic response to all three wavebands. However, careful analysis of a weaker *nph1* mutant revealed differential effects on phototropic sensitivity to B and G, implying the action of two distinct photosensory systems differing in relative sensitivity to these wavebands (Konjevic et al. 1992). While the identity of the photoreceptor(s) for these responses has

yet to be determined, B-dependent phosphorylation of a 120kDa membrane-associated protein shows similar kinetics and fluence dependence to phototropism and on this basis has been suggested to have an early role in phototropic signal transduction (Short et al. 1992). Furthermore, the level of this protein is normal in the *hy4* mutant, but severely reduced in *nph1* mutants (Reymond et al. 1992b, Liscum and Briggs 1995). It has therefore been suggested that the protein may be encoded by *NPH1*, and may represent a multichromophoric holoprotein with a specific role in the mediation of B, G and UV-A-stimulated phototropism (Liscum and Briggs 1995).

1.4. Light Signal Transduction

In many cases the activation of different photoreceptors may result in the same terminal response. For example, inhibition of stem elongation or the induction of anthocyanin biosynthesis may both be induced by phyA, phyB or cryptochrome. This suggests that unique signalling pathways for each photoreceptor must converge at some point. In contrast, activation of a single photoreceptor in many cases results in the induction of several diverse responses. These observations suggest that transduction of light responses is a relatively complex process, involving both convergent and divergent branching. Three main approaches have been used in recent investigations of light signal transduction. One approach has explored the possible involvement of various signalling systems known to be important in animals and bacteria. A second approach has involved the attempts to identify *cis*- and *trans*-acting factors required for photoregulation of certain genes. Finally, genes involved in signalling pathways have been pursued by the isolation of mutants which have impaired light responses.

1.4.1. Biochemical Approaches

The possibility that phytochrome has inherent protein kinase activity has received support both from biochemical studies (e.g. Wong et al. 1989, Biermann et al. 1994) and from sequence comparisons revealing homology of the C-terminal domain of phytochrome to bacterial and eukaryotic protein kinases (Schneider-Poetsch and Braun 1991, Thümmler et al. 1995). A role for protein phosphorylation in the early stages of B signal transduction has also been suggested (Reymond et al. 1992a). However, definitive evidence for protein kinase involvement in the action of either photoreceptor system is still lacking. Evidence for both phytochrome and blue-light regulation of G-protein activity has been obtained (Warpeha et al. 1992, Clark et al. 1993), and calcium has also been implicated in the mediation of responses to R (Shacklock et al. 1992). In possibly the most informative

approach to date, various signalling intermediates were assayed for their ability to substitute for phyA in the induction of anthocyanin accumulation and chloroplast development in hypocotyl cells of the phytochrome-deficient *aurea* mutant of tomato (Neuhaus et al. 1993, Bowler et al. 1994). The results from these studies suggest that at least two signalling pathways proceed from phyA, respectively involving cGMP and Ca^{2+}-calmodulin.

1.4.2. Photoregulation of Gene Expression

Promoter analyses of various genes have identified a number of regulatory sequences with roles in the mediation of light-regulated expression, including both positive and negative elements (Batschauer et al. 1994, Terzaghi and Cashmore 1995). Proteins which bind to these elements have also been identified, and in some cases, cloned (e.g. Kuhn et al. 1993, Sun et al. 1993, Menkens et al. 1994). While binding activity and/or expression of certain of these proteins has been shown to be influenced by light, clear evidence that they influence transcription has yet to be obtained (Batschauer et al. 1994, Terzaghi and Cashmore 1995).

1.4.3. Genetic Approaches

Two broad classes of mutant which appear to be involved in light signal transduction have been identified. The first class includes recessive mutants *hy5*, *fhy1* and *fhy3* in *Arabidopsis* (Koornneef et al. 1980, Whitelam et al. 1993) and the *hp-1* mutant of tomato (Peters et al. 1989) which, like photoreceptor mutants, have altered sensitivity to light but show normal development in darkness. The *fhy1* and *fhy3* mutants specifically affect responses to FR and are therefore presumed to lie in a pathway specific for phyA (Whitelam et al. 1993), whereas the *hy5* mutation confers reduced response to light of all wavelengths (Koornneef et al. 1980), suggesting it acts at or after the point at which phytochrome and cryptochrome signals converge (Chory 1992). The *hp-1* mutant is unique in conferring a light-hypersensitive phenotype, and HP-1 is suggested to control the amplification of the phytochrome signal or the input from a B receptor (Peters et al. 1992, Kendrick et al. 1994).

The second class consists of a broad and heterogenous group of mutants which display a somewhat de-etiolated phenotype in etiolated plants, including reduced stem elongation, increased leaf or cotyledon expansion, and expression of genes not normally expressed in darkness. These include *cop*, *det* and *fus* mutants of *Arabidopsis* and the *lip1* mutant of pea (e.g. Chory et al. 1989b, Deng et al. 1991, Frances et al. 1992, Misera et al. 1994). Of the genes identified in this manner, a subset are likely not to be directly involved in light

signal transduction, but may be more generally required for normal development in both the light and dark (e.g. *det2*, Li et al. 1996). However, others appear to be specifically required for repression of the de-etiolated phenotype and may function as components of one or more light-responsive "master switches" acting after the convergence of pathways from phytochrome and B receptors. Although epistasis studies have been used to place these genes in a regulatory hierarchy (Chory 1992, Ang and Deng 1994), it has not yet been established whether they have a structural or merely permissive role in signal transduction. However, certain structural features of COP1 (McNellis et al. 1994) and the nuclear localisation of DET1 and COP1 (Pepper et al. 1994, von Arnim and Deng 1994) suggest that these proteins may have a role in the dark-repression of photoregulated gene expression.

1.5. Aims and Scope of this Thesis

This thesis comprises an investigation of the genetic control of photomorphogenesis in the garden pea (*Pisum sativum* L.), and focuses particularly on the role of phytochrome. Pea has well-documented classical genetics (Weeden et al. 1993) and a long history of use in photomorphogenesis research (Went 1941, Parker et al. 1949, Hillman 1965, Nagatani et al. 1984, Abe et al. 1989), two features which make it well suited for use as an additional model species for the study of photomorphogenesis. In particular, its utility for biochemical studies has led to its use in the investigation of phytochrome and blue-light signal transduction (e.g. Datta et al. 1985, Warpeha et al. 1992, Short et al. 1992, Clark et al. 1993). Its caulescent habit and clear photoperiod response respectively distinguish it from *Arabidopsis* and tomato, and as a legume it is representative of a wide variety of economically important forage and crop species (Adams and Pipoly 1980, Summerfield and Roberts 1985). Also, the genetic control of processes such as photoperiodic flower induction (Murfet 1985) and gibberellin biosynthesis have been well-studied in pea (Ross 1994). Thus, in addition to the useful information likely to be directly obtained from characterisation of phytochrome-deficient mutants in pea, research in several related areas also stands to benefit from the availability of such mutants.

The following experimental chapters describe the isolation and characterisation of mutants deficient in phytochromes B (**Chapter 3**) and A (**Chapter 4**) and in phytochrome chromophore synthesis (**Chapter 5** and **6**). **Chapter 7** reports on investigation of a dominant mutant with a strongly light-hypersensitive phenotype. **Chapter 8** presents a final summary of results and a brief concluding discussion.

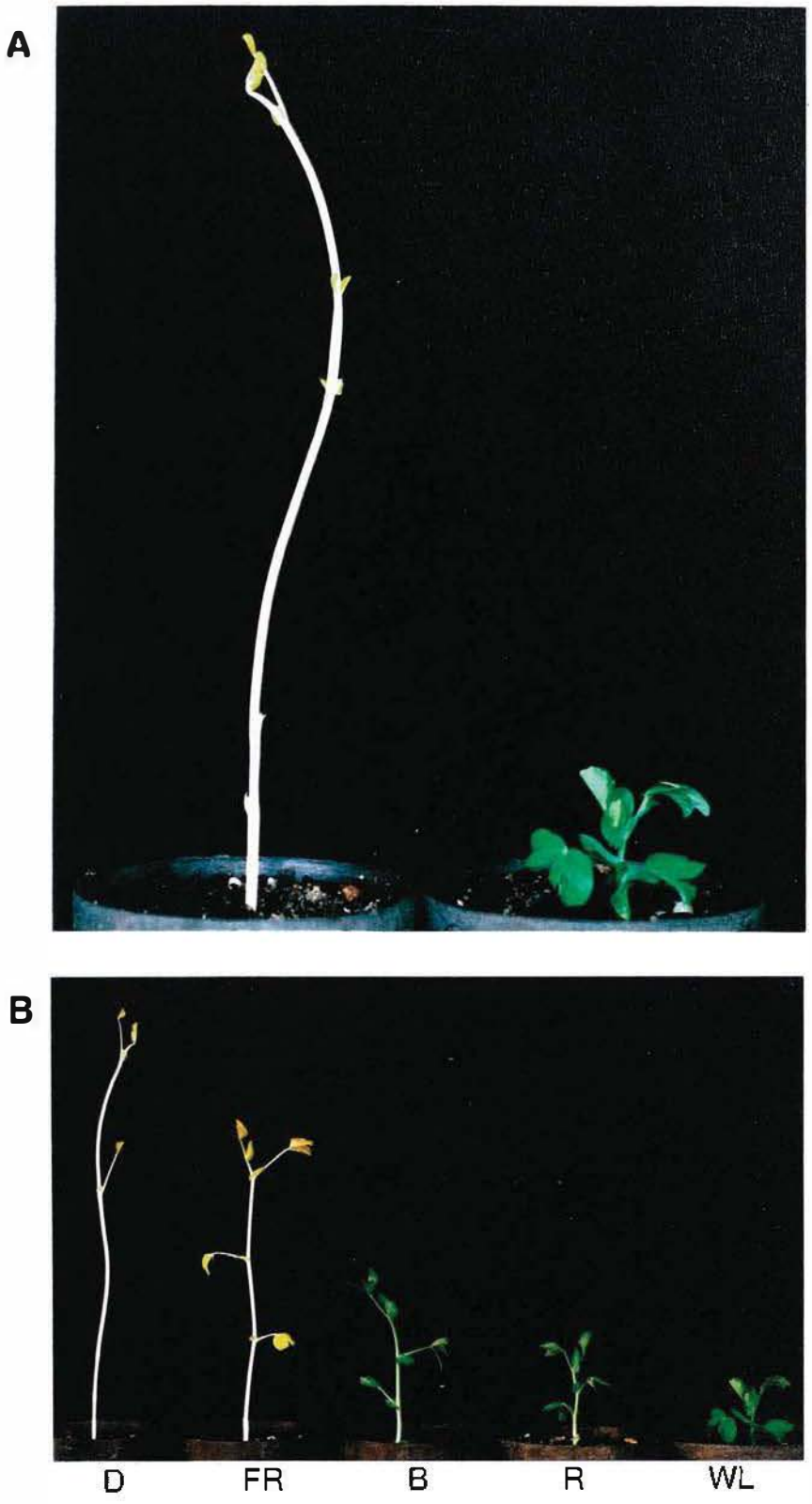

Figure 1.1. Effects of light on pea seedling development. A. Seedlings grown in complete darkness (left) and under continuous WL (150 μmol m^{-2} sec^{-1}). **B.** Seedlings grown in (from left) continuous darkness (D), FR (8 μmol $m^{-2}sec^{-1}$), B (10 μmol m^{-2} sec^{-1}), R (20 μmol $m^{-2}sec^{-1}$) or WL, at 20°C. All plants are 12 days old. The line shown is NGB5839, a GA_1-deficient dwarf mutant (*le*-3) in the cv. Torsdag background.

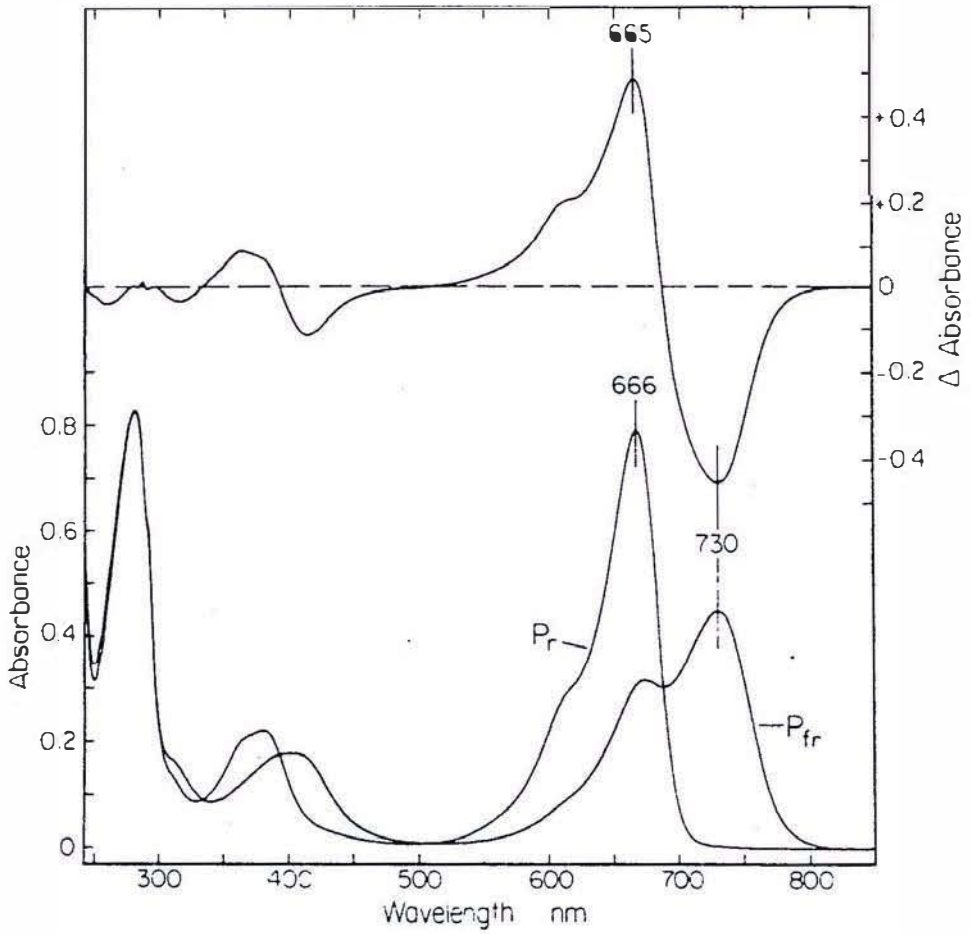

Figure 1.2. Absorbtion spectra for the P_r and P_{fr} forms of phytochrome. From Vierstra and Quail (1983).

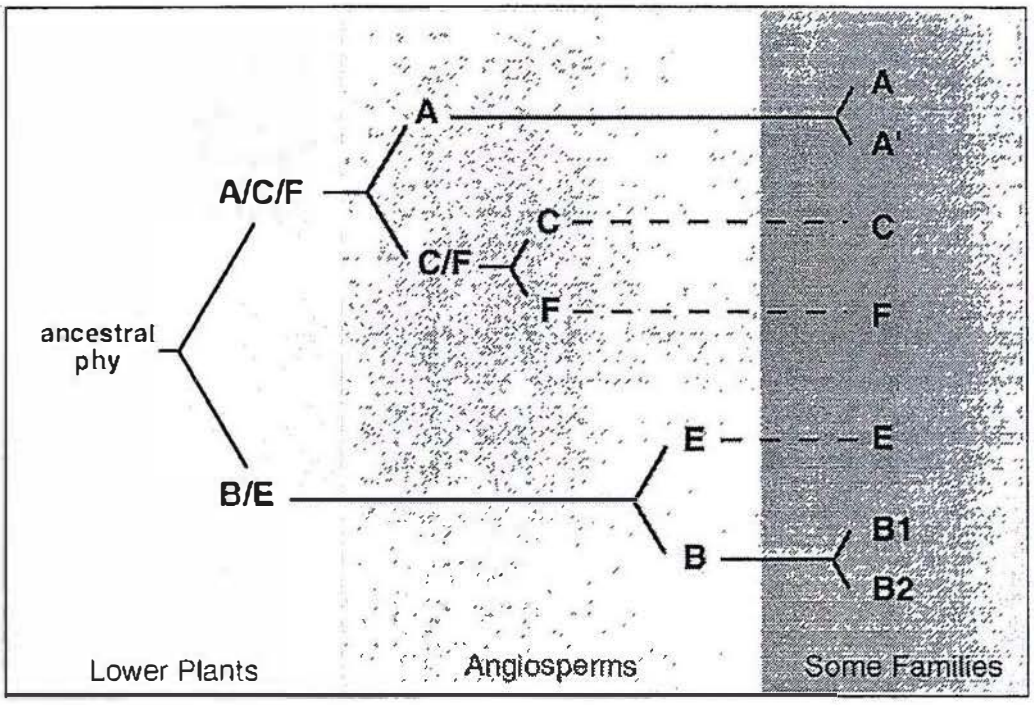

Figure 1.3. Diagram showing a summary of the phylogenetic relationships among the various phytochrome genes cloned from higher plants, based on data of Sharrock and Quail (1989), Clack et al. (1994), Mathews et al. (1995) and Hauser et al. (1995).

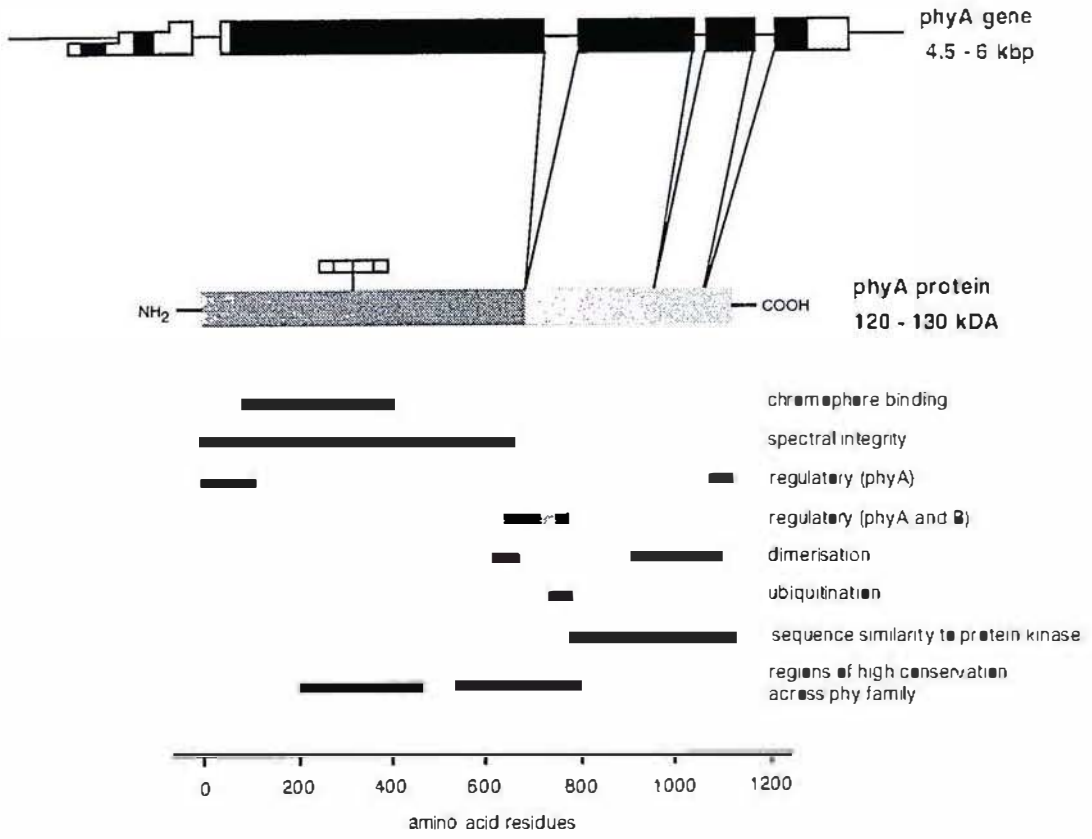

Figure 1.4. Diagrammatic representation of a generalized *PHYA* gene and encoded protein. In the diagram of the *PHYA* gene, exons are boxed and open reading frames shown in black. Multiple transcription start sites (found only in dicot genes) are indicated by steps in the first exon. In the diagram of the phyA protein, the chromophore is represented by the four rectangular boxes, and regions of functional significance (see text) indicated by the bars below.

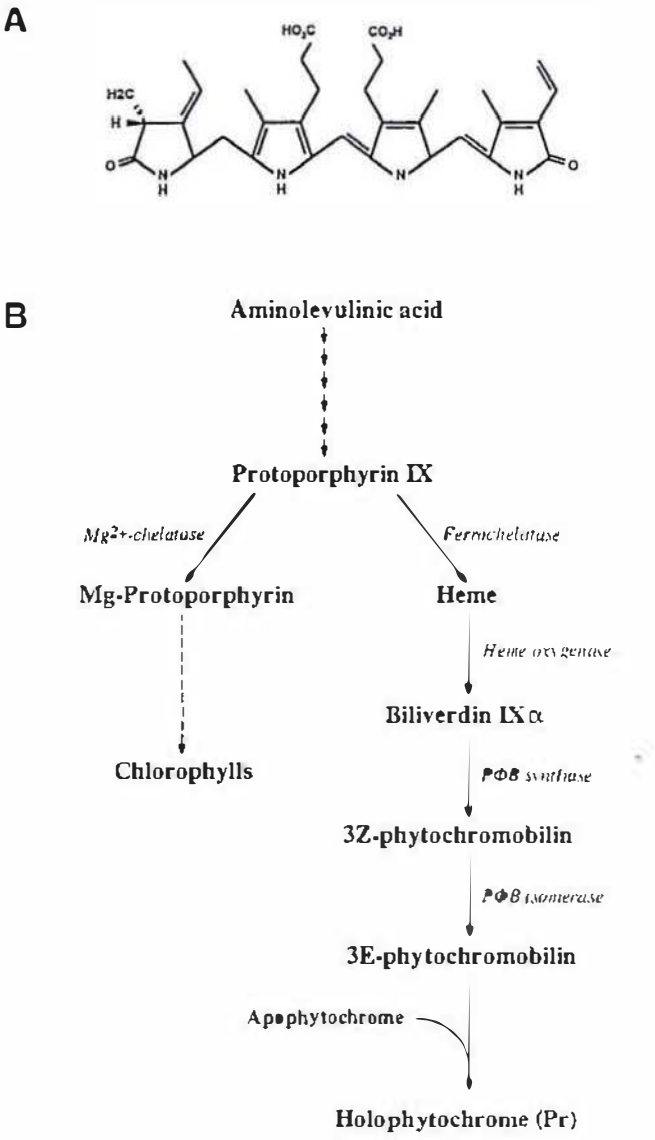

Figure 1.5. A. Structure of the phytochrome chromophore, 3*E*-phytochromobilin. B. Proposed pathway for phytochromobilin synthesis.

2. General Methods

2.1. Plant Husbandry

All plants grown in Hobart were grown in a standard potting mix of vermiculite and 9 mm dolerite chips (1:1, v/v), topped with 2-3 cm potting soil. Plants were grown in 14 cm diameter plastic pots, or at various densities in plastic tote boxes (30 x 40 x 12 cm) when it was not necessary for plants to be grown to maturity. Some experiments were performed in the Laboratory for Photoperception and Signal Transduction at RIKEN in Japan. These experiments are indicated in the text. Plants for these experiments were grown in plastic trays in pure vermiculite which had been fully saturated with water and then well-drained.

The seed coat of each seed was nicked before sowing and seeds were sown at a depth of approx 1.5 cm below the soil surface. Pots were transferred to the phytotron or growth cabinets within 24 h of sowing. Under normal glasshouse and growth cabinet conditions, emergence of seedlings through the soil surface was first apparent on the fourth day after sowing. In accord with the standard Hobart practice, lateral shoots were regularly removed from all plants. This was usually only necessary for plants grown under short photoperiods. For all experiments, only main shoots were scored. Counting of nodes commenced with the cotyledons as node 0. Above the cotyledons, pea seedlings produce two scale leaves before the first true foliage leaf at node 3. In general plants were watered every second day, although this was increased in frequency to every day in warmer weather or for plants at the flowering stage. Nutrient solution was supplied once weekly.

2.2. Growing Conditions

Two glasshouse environments were used. General-purpose glasshouse space received a natural photoperiod extended before dawn and after dusk with mixed incandescent and fluorescent light, to give a day-length of 18 h. The mean daily temperature in this glasshouse ranged from 13-21°C in winter and 17-30°C in summer. Phytotron space was used when controlled photoperiod conditions were required. Plants grown in the phytotron received an 8-h natural photoperiod and were then transferred to night-compartments where further light treatments were administered. The mean daily temperature range in the phytotron glasshouse varied from 16-24°C in winter to 17-27°C in summer, while the night compartments were maintained at 16°C throughout the year. Plants for all experiments conducted at RIKEN were grown in growth cabinets at 25°C.

2.3. Light Sources

Standard Hobart light sources were as follows; WL, 40W cool-white fluorescent tubes (Thorn, Australia); R, 40W red fluorescent tubes (Tungsram, Hungary); B, 40 W blue fluorescent tubes (Mitsubishi-Osram, Australia) wrapped with 2 layers of blue plastic film (cutting sheet 521C, Nakagawa Chemical, Tokyo, Japan); FR, 20W long-wavelength fluorescent tubes (FL20S-FR74, Toshiba, Tokyo, Japan) filtered through far-red plastic (FRF-700, Westlake Plastics, Lenni, PA, USA). Standard RIKEN light sources were as follows; R, 20W red fluorescent tubes (FL20S-RF, Toshiba); FR, FL20S-FR74 tubes wrapped in red and blue acetate film (red no. 22, blue no. 72, Tokyo Butai Shomei, Tokyo, Japan). Dim green safelight for all experiments in Hobart and at RIKEN was obtained from cool white fluorescent tubes (various) wrapped in blue, yellow and green plastic film (Nakagawa Chemical; cutting sheets 521C, 321C, 431C, respectively).

2.4. Indices of Growth and Development

A variety of plant characters were routinely scored in the various physiological experiments described in this thesis. In most experiments involving seedling de-etiolation responses, stem length was quantified as the length (Lx-y) between specified nodes x and y. Stem length was in some instances quantified as total plant height from soil surface to apical bud. The particular nodes chosen depended on the nature of the individual experiments. Leaf area was also estimated, as the product of the length and width of a leaflet from a specified leaf (either node 3 or 4 depending on the particular experiment). In some cases, the rate of leaf expansion was also measured, by a simple count of the number of expanded leaves at a given time, with the uppermost, partially open leaf given a decimal score between 0 and 1. The standard flowering characteristics recorded were node of flower initiation (NFI), node of flower development (NFD), time to first open flower, total number of nodes at senescence (TN), number of reproductive nodes (RN, equivalent to TN-NFI+1), and the flower/leaf relativity index (FLR) which essentially records how far behind the apical bud the first flower opens, and is a measure of the reproductive vigour of the plant (see Murfet 1985).

2.5. Immunoblotting

Approximately 0.2 g of 1 cm apical segments from 5-day-old etiolated seedlings were harvested on ice, frozen in liquid nitrogen and homogenised with approximately 0.02 g insoluble polyvinylpyrrolidone in 0.2 mL phytochrome extraction buffer (50% (w/v) ethylene glycol, 20 mM $NaHSO_3$, 52 mM 2-mercaptoethanol and 4 mM PMSF) containing

additional protease inhibitors leupeptin (2 μg/mL), pepstatin (1 μg/mL) and aprotinin (2 μg/mL) and 4 mM iodoacetamide (López-Juez et al. 1992). The homogenate was centrifuged at 15000g for 10 min. To this point, the extract was maintained at less than 4°C. The crude supernatant was collected, vortexed together with a 1:1 volume of 2x electrophoresis sample buffer and allowed to stand for 2 min at 90°C. Extracts were subjected to SDS-PAGE in a 6.5% gel, and proteins electroblotted onto a nylon filter (Fineblot, Atto, Tokyo) in 100 mM Tris, 192 mM glycine and 25% (v/v) methanol. Membranes were blocked in a series of Tris buffer/saline - Tween (TBST) solutions, essentially as described by López-Juez et al. (1992), with the exception that rabbit IgG was omitted from the second wash. Membranes were incubated with the primary antibody in TBST containing 1% (w/v) fat-free milk powder, for 1 h (phyA) or 2 h (phyB antibodies) at room temperature. After washing with TBST, membranes were incubated with a 1:5000 dilution of anti-mouse IgG-alkaline phosphatase conjugate (Protoblot, Promega) for 1 h at room temperature and stained according to the manufacturer's instructions.

The anti-pea phytochrome A monoclonal antibody mAP5 has been described previously (Nagatani et al. 1984). Monoclonal mAP11 (Konomi et al. 1987) was raised against a pea type 2 phytochrome purified from green plants (Abe et al. 1985). The mAT5 and mAT2 antibodies were raised against a C-terminal fragment of tobacco phytochrome B and shown to crossreact with the mAP11 purified type 2 pea phytochrome (phyB) but not with mAP5 purified type 1 phytochrome (phyA; López-Juez et al. 1992). All three of these phyB-specific antibodies were employed in this study.

2.6. *In Vivo* Spectrophotometric Assay for Phytochrome.

Photoreversible phytochrome content was measured with a dual-wavelength recording spectrophotometer (model 557, Hitachi, Tokyo) using 730 and 800 nm measuring beams. For each measurement, 8 apical segments (15 mm in length) from 5-day-old etiolated seedlings were harvested on ice, diced, and packed gently into a pre-cooled stainless steel cuvette, presenting a light path of about 4 mm. Phytochrome was photoconverted using saturating irradiances of R (45 sec) and FR (90 sec).

2.7. Chlorophyll Assay

For chlorophyll quantitation, two 6-mm discs were punched from the leaves or stipules and extracted in 2 mL of dimethylformamide for 24 h at 4°C. Chlorophyll levels in the extracts were determined spectrophotometrically according to Innskeep and Bloom (1985).

3. Mutants Deficient in Phytochrome B: The *lv* Locus

3.1. Introduction

As discussed in **Chapter 1**, the phytochrome family of photoreceptors in dicotyledonous plants is thought to consist of at least four distinct phytochrome types, corresponding to subfamilies A, B/D, C and E in *Arabidopsis*. Isolation of mutants deficient in specific phytochromes is currently the most effective way of defining their individual functions. Phytochrome B was the first phytochrome for which specific mutants were isolated (Somers et al. 1991, Nagatani et al. 1991). Study of these mutants established an important role for phyB in the mediation of responses to red light (R), including such responses as the R/far-red (FR) reversible low-fluence response (LFR) for inhibition of elongation in etiolated seedlings, the fluence-rate-dependent response to continuous R, and the responses to R:FR and end-of-day (EOD)-FR displayed by de-etiolated plants.

The pea *lv* mutant was originally identified on the basis of its elongated internodes when grown in white light (WL) (Reid and Ross 1988). Subsequent investigation of a single *lv* allele revealed a number of photomorphogenic defects, and established *lv* as the first known photomorphogenic mutant of pea (Nagatani et al. 1990). Etiolated *lv* seedlings showed reduced inhibition of elongation by R and WL but retained a normal response to FR. Etiolated seedlings also lacked the classic LFR for inhibition of stem elongation, while light-grown *lv* plants lacked a normal elongation response to EOD-FR (Nagatani et al. 1990). Mature *lv* plants had longer internodes and paler foliage than wild-type (WT) plants (**Figure 3.1**), indicating that the *LV* gene has a role throughout the life of the plant. These studies demonstrated the physiological similarity of the *lv* mutant to phyB-deficient mutants of *Arabidopsis*, cucumber and *Brassica rapa* (Somers et al. 1991, Devlin et al. 1992, López-Juez et al. 1992), and suggested that *lv* was a good candidate for a phyB-deficient mutant. However, immunoblotting analysis using a monoclonal antibody specific for a B-type phytochrome isolated from green pea tissue showed the *lv* mutant to have normal levels of both phyA and phyB apoprotein (Nagatani et al. 1990). It was therefore presumed that the *lv* mutant affected phyB signal transduction in some way.

Nevertheless, given the strong similarity between *lv* and known phyB-deficient mutants in other species, the possibility remained that *lv* might be a point mutation in a phyB gene, which specifically eliminated biological activity of the photoreceptor but did not alter its synthesis or stability. This possibility prompted a more thorough study of the *LV* locus. This chapter reports on these investigations, which essentially involved the

isolation of several new *lv* alleles, determination of their phyB status and detailed characterisation of their photophysiology. As an additional approach an M_2 population was screened under R in the hope of identifying novel loci involved in R perception. However, this was only pursued to a limited extent, and resulted only in the isolation of further *lv* alleles.

3.2. Results

3.2.1. Isolation of Additional *lv* Mutant Alleles

In addition to the original *lv* allele (line NEU3) investigated by Nagatani et al. (1990), another less severe allele (line R83) was identified by Reid and Ross (1988). Both of these mutant lines were included in the experiments described in this chapter. Two further mutant lines exhibiting aspects of the *lv* phenotype were also obtained. The elongated mutant Wt10895 was selected at Wiatrowo Plant Breeding Institute by Prof Dr W. K. Swiecicki after treatment of the dwarf (*le*) cultivar Paloma (Wt3527) with 200 r Nf (fast neutrons)/0.014% NEU (nitroso ethyl urea). The line 80m arose spontaneously in stocks of Hobart L80 (Lamm L30), and was first observed by Prof I.C. Murfet as having slightly elongated internodes and reduced leaf expansion under B. L80 is also a dwarf (*le*) line carrying the additional mutations *st* (reduced stipules) and *wlo* (upper leaflet surface waxless). Screening of a small M_2 population of EMS-treated cv. Torsdag (see **Chapter 4**) under R allowed the selection of two independent mutant lines, AF280 and AF300, which each showed increased stem elongation under R. Representative mutant and WT seedlings of all mutants are shown in **Figure 3.2.**

Each mutant was for tested for allelism with *lv* by crossing to a line homozygous for the originally isolated *lv* allele; either NEU3 (the original dwarf *le lv* line) or L232$^-$ (a tall *LE* line derived from the F_{10} of a cross between NEU3 and L107). The results in **Figure 3.3** and **Figure 3.4** show that F_1 plants in all cases displayed the *lv* phenotype, establishing that the elongated phenotype resulted from mutation at the *LV* locus. This result brings the number of *lv* mutant alleles to six, which are listed and given new designations in **Table 3.1.**

3.2.2. Inheritance of New *lv* Mutants

As expected, the F_2 of the cross between each new *lv* allele and its corresponding wild-type returned a clear segregation consistent with inheritance as a single recessive

mutation (**Figure 3.5**). A summary of all available segregation data for each of the *lv* alleles is presented in **Table 3.2**. In each case, the segregation ratio does not differ significantly from the expected 3:1, indicating that *lv* has no substantial effect on gamete production, fertilisation or embryo development.

The F_1 plants from crosses between *lv* mutants and their respective progenitor lines were in each case significantly longer than the corresponding WT (**Figure 3.6**). While these differences were relatively small, they do indicate incomplete dominance of the WT *LV* allele over the mutant alleles. On the basis of the internode length results in **Figure 3.6**, the degree of dominance of the *LV* allele over the *lv-1*, *lv-2*, *lv-4* and *lv-5* alleles was 85, 67, 74 and 53%, respectively.

3.2.3. Map Location of *lv*

Preliminary evidence from a small F_2 progeny from a cross of L232$^-$ (*lv-1*) to L111 (Marx's muti-marker line A875-55-0) indicated linkage between *LV* and the *WLO* locus in linkage group VI. F_2 segregation data for *lv* and group VI primary markers *wlo*, *na*, *Prx3*, *arg* and *pl* were obtained from three crosses as detailed in **Table 3.3**. These include two crosses involving the *lv-1* allele and one cross involving the *lv-4* allele. All individual segregations in **Table 3.3** are in accord with expectation ($P>0.05$). The joint segregation data revealed strong linkage of *lv* with *na* (<2cM), *wlo* (4cM) and *Prx3* (8cM), and moderate linkage with *arg* (26cM) and *pl* (26cM), with $P<10^{-6}$ and $<10^{-4}$, respectively. The fact that both *lv-1* and *lv-4* both map very close to *wlo* provides independent verification of the location of the *LV* gene. In addition to establishing the map location of *LV*, these results provide a picture of the linkage relationships in group VI which differs somewhat from that given in the currently accepted map. The data in **Table 3.3** generate the map shown in **Figure 3.7**, in which *wlo* located substantially closer to *Prx3*, *arg* and *pl* than the distance shown in recent maps (Weeden et al. 1991, 1993). The discrepancy relates specifically to the distance *wlo* to *Prx3* which is about one third of that shown on the latest map (Weeden et al. 1993). The distances for *Prx3-arg* and *Prx3-pl* are consistent with recent maps. Moreover, the data for *wlo-pl* and *wlo-arg* are entirely consistent with values obtained from very large data sets by Lamprecht (1961), Marx (1981) and Marx (1982). In the absence of multi-point data, the map position of *na* remains unclear. The 2-point data in **Table 3.3** place *na* and *lv* in close proximity and imply that *na* may lie between *wlo* and *pl*, as shown in the tentative map of Marx (1981).

3.2.4. Spectral Sensitivity of *lv* Mutants

Like the originally isolated *lv-1* mutant, all five new *lv* mutants were substantially longer than their respective WT lines in continuous R and W, but were no longer under FR (**Figure 3.8**). In general, there is considerable variation in the relative response of standard pea cultivars to monochromatic light, and variation in the response of the WT lines used in this study made it difficult to assess the relative strengths of the different mutant alleles. In particular, *le* dwarf cultivars show a proportionately greater inhibition of elongation by light than do WT tall (*LE*) lines (Reid 1988). This is at least partly the reason that *lv-5* appears less severe than *lv-1* or *lv-4* (**Figure 3.8**). However, on the same genetic background (cv. Sparkle) *lv-2* was clearly less severe than *lv-1*, as previously reported (Reid and Ross 1988). The *lv-1* and *lv-4* mutations appeared similar in relative severity under R. In addition, a difference in the response of *lv-1* to high fluence white light was apparent. The other three mutants on a dwarf background were approximately the same length in R as in WL and longer in FR, whereas *lv-1* was longer in WL than in R or FR (**Figure 3.8**, Nagatani et al. 1990). The close linkage of both *lv-1* and *lv-4* to *wlo* makes it unlikely that this difference in the *lv-1* phenotype might in part result from translocation or otherwise altered position of the gene. However, it cannot yet be ruled out that the difference may represent a background effect.

In order to examine the relative severity of the various *lv* alleles, each mutant line was backcrossed to cv. Torsdag, and segregation was observed in the resultant F_2 populations grown under WL (**Figure 3.9**). In this case, L232$^-$ was used as the source of the *lv-1* allele, as this background is substantially closer to cv. Torsdag than is the original *lv-1* background, cv. Sparkle. Although the single backcross did not eliminate background effects, it is apparent that the *lv-2* allele is less severe than *lv-1*, and that *lv-3* is probably the least severe. Alleles *lv-4* and *lv-5* both appeared relatively strong, while *lv-6*, although showing similar elongation to *lv-5* (**Figure 3.9**) was somewhat less severe in terms of leaf development (see **Figure 3.2**). These results suggest that *lv-1*, *lv-4* and *lv-5* should be the alleles of choice for use in the production of double mutants and for further physiological studies. Continued back-crossing of all *lv* alleles into the cv. Torsdag background will be necessary to provide a clearer picture of their relative phenotypic severity.

3.2.5. Phytochrome Levels in *lv* Mutants

The anti-pea phytochrome A antibody mAP5 detected a band of ≈121 kD at uniform intensity in extracts from WT and all *lv* mutants (**Figure 3.10A**, H. Hanzawa, pers. comm.).

While mAP11-immunopurified pea phyB was previously reported to migrate as a single band of 115 kD (Abe et al. 1989), the antibody detected two bands of about 116 kD in crude and partially enriched extracts from pea embryonic axes (Konomi et al. 1987). **Figure 3.10B** shows that in crude extracts from etiolated wild-type and *lv-1* seedlings, mAP11 also recognised two bands, at 117 and 115 kD, consistent with these previous reports. The same two bands were recognised by another anti-phyB monoclonal antibody mAT5 (**Figure 3.10B**). However, corresponding bands were not detected in extracts from *lv-2*, *lv-3* or *lv-4* plants by mAP11 or mAT5 (**Figure 3.10B**) despite uniform non-specific staining. An identical result was also obtained with the weakly staining mAT2 antibody (data not shown). The absence of both the 115 and 117 kD band in the mutants also provides evidence that the two bands derive from a single phytochrome and not from cross-reactivity to more than one phytochrome species.

These results confirm the normal presence of phyB-like apoprotein in *lv-1* reported by Nagatani et al. (1990) and clearly show this phytochrome to be absent in the *lv-2*, *lv-3* and *lv-4* mutants, or present below the detection limit. Recently, the *lv-5* has also been shown to be effectively null for this phyB apoprotein, whereas *lv-6*, like *lv-1*, retains WT level of the apoprotein (H. Hanzawa, pers. comm.). The fact that phyB was not detected in *lv-2*, despite its clearly leaky phenotype, does however suggest that the minimum level of phyB required for detectable biological activity is below the limit of sensitivity for the immunoblotting procedure employed.

3.2.6. Light Responses of Etiolated *lv* Seedlings

On the basis of results from mutants in several species, including those presented above for pea, it is generally regarded that the primary function of phyB is the sensing of R (Somers et al. 1991, McCormac et al. 1993, van Tuinen et al. 1995b). Furthermore, it is well known that different components of the seedling response to R can be distinguished on the basis of FR reversibility or dependence on fluence rate (Mancinelli 1994). To identify those components which are altered in the *lv* mutants, the responses of *lv* mutants to R was examined in greater detail. The data presented in **Figure 3.11** are those for *lv-1* and *lv-2*, on the same genetic background. Although the *lv-1* mutant is not, strictly speaking, phyB deficient, under the R conditions used it behaves similarly to the *lv-4* mutant (results not shown), which is severely deficient in the phyB apoprotein (**Figure 3.10**). The isogenic comparison between *lv-1*, *lv-2* and cv Sparkle was therefore preferred over the use of alleles on different backgrounds.

Saturating R pulses given every 4 h to WT plants resulted in strong inhibition of elongation relative to dark-grown plants. This inhibition was partially reversible by FR, to the level of plants given FR pulses alone (**Figure 3.11B**). The *lv-1* mutant appeared virtually null for this FR-reversible component, while the the *lv-2* mutant was clearly leaky. The residual, non-reversible component was retained in both mutants (**Figure 3.11A**). A directly comparable experiment examined the R fluence rate response in *lv-1* and *lv-2* mutants (**Figure 3.11B**). Elongation of WT plants was inhibited by continuous R in a fluence-rate dependent manner over a range in fluence rate of approximately 10^{-3} - 10^{-1} µmol m^{-2} sec^{-1}. Mutants *lv-1* and *lv-2* both showed a reduction in this response. Although it was not possible to obtain fluence rates lower than about $4x10^{-4}$ µmol m^{-2} sec^{-1}, the convergence of response curves for WT and *lv* mutant plants suggests that the mutants respond normally to R at lower fluence rates. Furthermore, this response to continuous very low fluence R was similar in extent to the non-FR reversible component seen in the pulse experiment (**Figure 3.11A**).

The results for leaflet expansion (**Figure 3.11C** and **D**) were essentially similar to those for stem elongation. The *lv* mutants lack the FR reversible component of the response to R pulses (**Figure 3.11C**) and the fluence-rate-dependent component of the response to continuous R (**Figure 3.11D**). WT plants showed a small response to very-low fluence-rate R for leaflet expansion, and this was retained in both mutants. Whereas *lv-1* was clearly less responsive than *lv-2* with respect to stem elongation (**Figure 3.11A** and B), leaf expansion responses in *lv-1* and *lv-2* plants were very similar, indicating that the level of phyB present in *lv-2*, though sufficient to inhibit elongation, is not sufficient to promote leaflet expansion. More generally, this suggests that there may be differences in the threshold level of phyB required for induction of the various developmental processes controlled by this phytochrome.

3.2.7. Light Responses of De-Etiolated *lv* Seedlings

Although the primary function of phyB thus appears to be the sensing of R, in pea (**Figure 3.8** and **3.11**) as in other species (Smith 1995), phyB also plays a role in the detection of FR in mature plants. In these responses, the effect of FR derives from a reduction in the proportion of phyB as P_{fr} (Smith 1995). One such response, involves elongation in response to a brief exposure to FR at the end of the day (the EOD-FR response). This response was previously shown to be lacking in the *lv-1* mutant (Nagatani et al. 1990). The addition of supplementary FR to background WL also causes an increase in stem elongation and a decrease in leaflet/cotyledon area (the so-called "shade-avoidance" response) in many plants, including pea. Various studies of phytochrome-deficient mutants have

demonstrated an important role for phyB in this response (Whitelam and Smith 1991, Devlin et al. 1992). The responses of the *lv-1* mutant to R:FR were therefore examined, employing various R:FR ratios established by supplementing light from cool-white fluorescent tubes with light from incandescent globes.

WT plants showed a small but typical shade-avoidance response, in which stem elongation increased in a roughly linear manner with increased FR content of the incident light (**Figure 3.12**). Mutant *lv-1* plants were longer than WT under all R:FR ratios used, but maximum inhibition of elongation occurred in light with the lowest R:FR (**Figure 3.12**). The overall increase in elongation from lowest to highest R:FR in *lv-1* was thus in marked contrast to the decrease shown by WT plants, indicating particularly that high R:FR was much less effective at inhibiting elongation in *lv-1* than in WT plants, and that the added FR actively inhibited elongation in *lv-1* plants. Leaflet area in WT plants was increased by both high and low R:FR ratios, with intermediate ratios being less effective (**Figure 3.12**); again suggesting an active role for FR. Both high and low R:FR ratios also increased leaflet area in *lv-1*, but to a lesser extent than in WT plants, particularly at high R:FR. Rate of node development is another important developmental characteristic in pea, which is substantially increased in light-grown relative to etiolated seedlings. Although the differences in rate of node development between WT and *lv-1* were less clear than for elongation and leaflet area, a similar trend was observed (**Figure 3.12**).

The use of unfiltered incandescent globes for FR supplementation of WFL is less than ideal however, as other regions of the spectrum in addition to FR are also supplemented. The R:FR ratio response of *lv* mutants was therefore re-examined, using two identical WFL sources and supplementation with monochromatic FR. This time three *lv* alleles (*lv-1*, *lv-2* and *lv-4*) were included, to test the possibility that the results from the previous experiment reflected a specific abnormality of the *lv-1* allele. Under these conditions, *lv-1* plants again showed inhibition of elongation and increased leaflet area under low R:FR, as did *lv-4* plants (**Figure 3.13**).

3.2.8. Role of PhyB in Photoperiodism

Garden pea is a quantitative long-day plant (LDP), flowering earlier in long photoperiods (LD) than under short photoperiods (SD). Because R/FR reversible phytochrome effects have been observed in connection with photoperiodic induction of flowering in pea (Reid and Murfet 1977) as in many other species, the association of phyB deficiency with loss of classic R/FR reversible low-fluence responses (**Figure 3.10, 3.11**) suggested that phyB might play a role in photoperiodic flower induction in pea. This suggestion is supported by

the early-flowering behaviour of the *hy3* (*phyB*) mutant of *Arabidopsis* (Goto et al. 1991). It was therefore of interest to examine the effect of *lv* mutations on flowering and photoperiod responses.

Growth and flowering behaviour of WT and *lv-1* plants were compared under 8-, 12-, 16- and 24-h photoperiods provided as 8 h natural daylight extended with weak incandescent light. These are the standard conditions employed for examination of photoperiod responses, since the low fluence rate extensions do not contribute significantly to photosynthesis and thus do not introduce any confounding differences across the range of photoperiods. **Figure 3.14** shows that there was no significant difference in the node of flower initiation (NFI) between WT and the *lv-1* mutant in 24-h conditions, with both lines flowering at about node 14. This indicates that phyB is not required for the detection of a photoperiod extension. However, in the 8 h photoperiod, *lv-1* plants intiated flowering significantly earlier (NFI=15.6) than WT (NFI=20.7; **Figure 3.14**). On the basis of NFI, it therefore appears that the *lv-1* mutation imparts a marked reduction in the ability to respond to photoperiod, flowering only 1.5 nodes later ($P<0.001$) in 8h than in 24h (compared with a 6 node delay in the WT). However, *lv* plants aborted flower initials in the shorter photoperiods, and thus retained a much stronger photoperiod response for flower development than for flower initiation (**Figure 3.14**). The retention of an effect of a 16-h photoperiod extension on other characters such as internode length, and the flower/leaf relativity index (FLR) is shown in **Figure 3.15**, and again indicates that *lv-1* plants retain some sensitivity to photoperiod. **Figure 3.15** also shows that these effects are not restricted to the *lv-1* allele, but are also seen for *lv-5*.

To further investigate whether the altered flowering behaviour was a general effect of phyB deficiency or a specific effect of the *lv-1* allele, *lv* mutant lines were backcrossed to cv. Torsdag and segregation of the mutant allele observed in F_2 populations grown under SD conditions. The results in **Figure 3.16** show that clear segregations for NFI were only obtained in F_2 populations from crosses involving *lv-1*, *lv-5* and *lv-6*. The mean values for NFI of WT and *lv* segregates in **Figure 3.16** show that like *lv-1*, *lv-2* and *lv-5*, *lv-3* also promoted flowering in SD, whereas *lv-2* and *lv-4* had no significant effect. Importantly, the three alleles showing a clear effect on NFI were either already in a Torsdag background (*lv-5* and *lv-6*) or had been been back-crossed to Torsdag once already (*lv-1*), whereas the other alleles were crossed in from quite different backgrounds. For this reason it seems probable that the early flowering in these lines is a real and general effect of phyB deficiency on flowering, and the failure to detect this effect in *lv-2* and *lv-4* results from background effects. The distributions for NFI in the crosses involving *lv-2* and *lv-4* certainly do suggest some evidence for segregation of an additional flowering difference. In

any case, these suggestions will be easily tested during the course of further backcrossing of all *lv* alleles into the Torsdag background.

3.2.9. Effect of phyB on Phytochrome Reaccumulation

High levels of *PHYA* mRNA and phyA apoprotein are present in dark-grown pea seedlings and are rapidly depleted on exposure to R. Subsequent return to darkness results in an eventual re-accumulation of both mRNA and apoprotein (Clarkson and Hillman 1967, Otto et al. 1984). The re-accumulation of *PHYA* mRNA is hastened by FR treatment, whether given directly after the R treatment or after an intervening dark period of up to 16 h (Furuya et al. 1991). These results indicate that the regulation of phyA abundance involves a rapid initial destruction on conversion to P_{fr} and a subsequent persistent repression of further phyA synthesis which may be alleviated by FR treatment, in a manner consistent with control by a stable phytochrome. This pattern of control has also been reflected in studies of *PHYA* expression in oat (Colbert et al. 1985, Lissemore and Quail 1988) and regulation of phyA levels under a variety of conditions in several other species (Schäfer 1978, Carr-Smith et al. 1994, Quail 1994).

To examine whether this stable phytochrome might be phyB, the effects of the *lv-4* mutation on the destruction and subsequent re-accumulation of spectrophotometrically detectable phytochrome were investigated. The *lv-4* mutation was chosen because at the time the experiment was conducted it was the most severe of the available *lv* mutations causing deficiency in the phyB apoprotein. **Figure 3.17** shows that phytochrome levels in etiolated WT and *lv-4* plants were similar, and showed similar rates of depletion in R, to about 15% of the level in etiolated plants after 4 h R. WT plants returned to darkness after 4 h R showed a gradual re-accumulation of phytochrome to about 45% after 48 h. However, *lv-4* plants showed more rapid re-accumulation, to about 66% after 48 h (**Figure 3.17a**). A pulse of FR given immediately following R treatment also hastened re-accumulation in both WT and *lv-4* plants, to about 75% after 48 h in each case (**Figure 3.17c**). However, clear differences in morphology became apparent between WT and *lv* mutant plants grown under this regime, and as in vivo spectrophotometric measurements are sensitive to changes in the optical properties of the sample, the effect of an additional 1 h R treatment given 24 h after initial transfer to darkness was tested (**Figure 3.17d**). This treatment re-depleted phytochrome in both lines to the same level but had no visible effect on apical morphology in either genotype, indicating that the apparent difference in phytochrome content after 24 h darkness was not due to a morphological difference.

3.3. Discussion

Phytochrome B in *lv* Mutants

Previous characterisation of a single allele showed the *lv* mutant (*lv-1*) to be phenotypically similar to the phyB-deficient mutants *lh* in cucumber and *hy3* (*phyB*) in *Arabidopsis*. All three mutants show reduced inhibition of elongation by R and lack an elongation response to EOD-FR (Adamse et al. 1987, López-Juez et al. 1990a, 1992, Nagatani et al. 1990, 1991). Contrary to expectation, the *lv-1* mutant was found to have normal levels of phyB apoprotein (Nagatani et al. 1990), and thus could not provide a proven link between phytochrome B and red light responses in pea. However, the isolation of additional *lv* alleles (**Figure 3.3**) which are clearly deficient in a phyB apoprotein (**Figure 3.7**) confirms that the phenotypic syndrome displayed by the *lv-1* mutant does result from a specific loss in the activity of a B-type phytochrome. The antibodies used to detect pea phyB were raised against a light-stable phytochrome isolated from green pea tissue (Nagatani et al. 1987). The limited amount of amino acid sequence data available for this phytochrome (69 residues, Abe et al. 1989) indicates a greater similarity to B-type phytochromes (86% to phyB, 81% to phyD) than to phyA (65%), C (61%) or E (70%) of *Arabidopsis* (Sharrock and Quail 1989, Clack et al. 1994). It is now clear that in many higher plant species the phyB subfamily may have at least two members (Mathews et al. 1995, Pratt et al. 1995). However, as the existence of more than one B-type phytochrome in pea has yet to be demonstrated, the missing phytochrome will be referred to in an unqualified manner as phyB, although the exact molecular identity of this phytochrome is not yet known.

The identification of phytochrome-deficient *lv* mutants now makes it clear that the original *lv-1* mutation must alter the function of the photoreceptor without detectable alteration to its size, and suggests that *LV* may be a phyB structural gene. However, whether the protein-positive *lv-1* and *lv-6* mutations impart additional, abnormal activity to the phyB molecule is not clear, and care is advised in the use these mutants as effective phyB nulls. In any case, molecular analysis of *lv-1* and *lv-6* should be useful in identifying residue(s) important for phyB function. Similar loss-of-function mutations have been isolated in both the *PHYA* and *PHYB* genes of *Arabidopsis*, and have defined several residues critical for phy activity which are mostly located within a 160-amino-acid region of the C-terminal domain (Xu et al. 1995, Wagner and Quail 1995).

The incomplete dominance of the WT *LV* allele (**Figure 3.6**) is in keeping with previous reports that phytochrome-deficient mutants of *Arabidopsis* show a partially dominant

phenotype (Koornneef et al. 1980, Whitelam et al. 1993). Haplo-insufficiency of photoreceptor structural genes has also been reported for phyA (Whitelam et al. 1993), phyB (Wester et al. 1994) and for the blue light receptor cryptochrome (Koornneef et al. 1980, Ahmad and Cashmore 1993), and thus appears to be a more general phenomenon. It suggests that photoreceptor concentration is rate-limiting for photoreceptor activity, and therefore that regulation of phy level may be a critical factor in the regulation of phy activity. Consistent with this notion, it has been reported that the elongation phenotype of phyB overexpressing transgenic *Arabidopsis* varies in a linear manner with *PHYB* copy number (Wester et al. 1994).

Role of Phytochrome B in Etiolated Seedlings

The effects of *lv* mutations on red light responses of etiolated pea seedlings (**Figure 3.11**) indicate that phyB mediates the classic LFR for stem elongation, induced by R pulses or low fluence rate continuous R. These results are for the most part consistent with those obtained in similar experiments with the *Arabidopsis phyB* mutant (McCormac et al. 1993, Reed et al. 1994). Furthermore, the threshold and saturation fluence rates (approximately $4x10^{-4}$ and $4x10^{-1}$ $\mu mol\ m^{-2}\ sec^{-1}$, respectively) for the fluence-rate dependent component of the response to continuous R appear roughly similar between the two species (**Figure 3.11B**, Reed et al. 1994). However, these results also highlight possible differences between the pea and *Arabidopsis* systems, in the form of responses which are clearly apparent in pea but which are absent or undetectable in *Arabidopsis*. Firstly, a very low fluence rate response is clearly evident in pea, in which seedling stem elongation may be inhibited 20-30% by brief FR or R/FR pulses or by very low fluence rate continuous R (**Figure 3.11A** and **B**). However, in *Arabidopsis*, these same treatments appear to have little or no effect (McCormac et al. 1993, Reed et al. 1994). Further experiments will be necessary to determine whether this response corresponds to the previously described very low fluence response (VLFR, Mancinelli 1994) or is simply a more sensitive LFR. Secondly, inhibition of hypocotyl elongation by continuous R at high fluence rate is completely lacking in the *Arabidopsis phyB* mutant (McCormac et al. 1993, Parks and Quail 1993, Reed et al. 1994), whereas all *lv* mutants, although longer than the WT under R, still show a strong inhibition of elongation relative to plants grown in darkness (**Figure 3.8** and **3.11B**), particularly over the first 2-3 d after emergence.

While none of the *lv* mutants has conclusively been shown to be null, plants carrying the severe *lv-1* allele appear completely lacking in the FR-reversible component of the response to R pulses, yet retain a response to continous high fluence rate R (cf. **Figure 3.11A** and **B**). This is also true for the *lv-4* allele (results not shown), which has no detectable

phyB apoprotein (**Figure 3.10**). The failure to find clear residual effects of R on hypocotyl elongation in phyB null mutants of *Arabidopsis* has led to the conclusion that phyB may be the only phytochrome mediating elongation responses to R (McCormac et al. 1993, Parks and Quail 1993). Our results indicate that this is obviously not the case in pea, and raise the question of which phytochrome(s) mediate the responses to R retained in the *lv* mutant. It is becoming increasingly clear from recent work with *Arabidopsis* that the non-FR reversible low fluence rate components of the R-induced inhibition of hypocotyl elongation, seed germination and *CAB* induction are controlled by phyA (Reed et al. 1994), and it seems highly probable that this is also the case in pea. However, evidence for phyA-controlled inhibition of elongation under higher fluence rate R is somewhat limited, and is provided mainly by the fact that the *Arabidopsis phyA phyB* double mutant appears slightly longer than *phyB* single mutant under continuous R (Reed et al. 1994). The phyA-deficient *fri*^{1}mutant of tomato is also slightly longer than WT under R, but it is unclear whether this is a real response to R since a difference is also apparent in dark-grown plants (van Tuinen et al. 1995a). Phytochrome A may therefore have a role in the mediation of the residual response to R seen in the *lv* mutants, but it is also possible that additional phytochrome(s) may be involved. Further investigation of this problem will obviously require the isolation of a pea mutant deficient in phyA.

However, interesting recent evidence for the presence of more than one phyB-like, R-sensing phytochrome has come from the *tri* mutant of tomato (van Tuinen et al. 1995b). This mutant lacks one of the two phyB-like phytochromes present in tomato (Pratt et al. 1995) and shows reduced sensitivity to R, but in contrast to both *Arabidopsis phyB* and pea *lv* mutants the R-insensitivity of *tri* plants is restricted to the first two days of exposure to light. Mature light-grown *tri* plants retain normal responses to EOD-FR treatment and have an essentially wild-type appearance (van Tuinen et al. 1995b). Although duplication and differentiation of phyB genes appears to have occurred relatively recently (Matthews et al. 1995, Pratt et al. 1995), and homology between B-type phytochromes from different species is therefore no guarantee of orthology or conservation of function (in any but the most general sense), the evidence from tomato at least raises the possibility that seedling responses to R may in some cases be controlled by at least two phytochromes other than phyA. In tomato, these would appear to mediate overlapping but temporally distinct responses to R, one early and temporary (missing in the *tri*^{1} mutant) and another later and persistent (responsible for control of the EOD-FR response). It is therefore possible that the response to R retained in all *lv* mutants (**Figure 3.8** and **3.11B**) may similarly involve the action of another B-type phytochrome. In any event, the strength of this residual R effect suggests that pea may be a useful species for the identification of mutants lacking this response.

Role of Phytochrome B in De-Etiolated Seedlings

Taken together, the results in **Figure 3.12** and **3.13** show that the response to R:FR in pea is a composite response, involving promotion of de-etiolation (inhibition of stem elongation, increased leaflet expansion) by both R-rich and FR-rich light. The role of phyB in this response is clearly the promotion of de-etiolation by R-rich light, as in all cases the expression of the *lv* mutant is greatest under these conditions (**Figure 3.12**). For promotion of leaflet expansion, the response to FR-rich light is clearly visible in WT plants, whereas for inhibition of elongation it is only visible in the absence of phyB. This conflicts somewhat with the prevailing assumption that the response to low R:FR only essentially involves the action of phyB, reflecting relief of the inhibition of elongation imposed by $P_{fr}B$. Furthermore, it suggests that the degree of "shade-avoidance" displayed by the plant under low R:FR is determined by antagonistic effects of FR; an enhancement of shade avoidance mediated by a reduction in $P_{fr}B$, and a promotion of de-etiolation by FR-rich light. Although the effectiveness of FR in the induction of de-etiolation is well known, it has generally been considered that this response to FR is lost with full de-etiolation. Results similar to those found in the present study were reported from a comparison of the R:FR responses of a number of photomorphogenic mutants and phytochrome A overexpressors (McCormac et al. 1991, 1992). These authors identified not only a negative response to low R:FR, but also a temporal change in the sensitivity to low FR. A negative response to low R:FR was detectable very early in seedling development, but was replaced by a more normal "positive" shade-avoidance response after longer periods of de-etiolation. The absence of phyB magnified the negative effect of low R:FR and extended its duration (McCormac et al. 1992).

The large amount of phyA present in etiolated seedlings suggests that this phytochrome may be predominantly responsible for de-etiolation responses in the time directly following transfer to light. In *Arabidopsis*, the main role of phyA in etiolated seedlings appears to be the detection of FR (Parks and Quail 1993, Whitelam et al. 1993). It is therefore probable that the promotion of de-etiolation by FR seen in pea (**Figure 3.12** and **3.13**) and in *Arabidopsis* (McCormac et al. 1992) is mediated by phyA. Normally, upon de-etiolation, phyA is depleted and other light-stable phy species become predominant, with the response to FR overridden by a stronger R/FR reversible response mediated by phyB. However, in the absence of phyB, the de-etiolation response to FR persists in the green seedling, as in the *lv* and *Arabidopsis phyB*mutants (**Figure 3.12** and **3.13**, McCormac et al. 1992).

Role of Phytochrome B in Photoperiodism

Reid and Murfet (1977) identified two responses involved in photoperiodic flower induction in pea; one in which flowering was promoted by long exposures to FR-rich light (a so-called "high irradiance response" or HIR), and another in which flowering was promoted by shorter exposures to R. A small but significant reversal by FR of this R response was also detected, suggesting the possible presence of a response in the LFR mode. Similar findings have also been reported for a number of other LDP (Vince-Prue 1994). However, in most cases the night-break effect has been found to be much weaker than the HIR, thus suggesting the HIR to be the predominant phytochrome response underlying LDP photoperiodism (Vince-Prue 1994). Since flowering in *lv* plants is promoted to the same extent as WT plants in extended SD (**Figure 3.14** and **3.15**), phyB does not appear to be involved in the HIR. This is consistent with results from seedling photoresponses in which *lv* plants retain a normal response to FR (**Figure 3.8**).

The possibility that phyB might be involved in the R night break effect is suggested by the apparent similarity of the small FR-reversible component to a classical LFR. However, the finding that *lv* plants are early flowering under SD (**Figure 3.14** and **3.15**) indicates that phyB has an *inhibitory* role in the regulation of flower induction, rather than a promotory function as might be expected for a R/FR reversible night-break acting through $P_{fr}B$. This inhibitory role for phyB is supported by results from phyB-deficient mutants in *Arabidopsis* (Goto et al. 1991) and *Brassica* (Devlin et al. 1992), which also flower earlier than WT under non-inductive conditions, whether given as SD (Goto et al. 1991) or as LD of high R:FR (Halliday et al. 1994). These results appear to rule out the suggestion that phyB mediates the promotory effect of R night-breaks in LDP, unless an inhibitory activity of P_rB is invoked. An active role for P_rB is not without precedent (Liscum and Hangarter 1993, Shimomura et al. 1994, Robson and Smith 1996). However, the retention of promotory responses to night breaks (Reed et al. 1994), to low R:FR (Halliday et al. 1994) and to EOD-FR treatment (Bagnall et al. 1995) in *phyB* mutants indicates that even if phyB does have a promotory role in *Arabidopsis*, it is not the sole photoreceptor responsible for LFR-like promotory responses.

As in LDP, physiological studies of photoperiodism in short day plants such as *Lemna*, *Pharbitis* and *Chenopodium* have also revealed two distinct responses, involving both promotory and inhibitory effects of FR (Cumming et al. 1965, King et al. 1982, Takimoto and Saji 1984, Lumsden et al. 1987). In contrast to LDP however, the majority of evidence indicates that the predominant phytochrome response in SDP photoperiodism is a classical LFR (Vince-Prue 1994), in which interruption of an inductive long night by R

inhibits flowering in a FR reversible manner. Attempts have been made to correlate these responses with specific pools of phytochrome on the basis of apparent differences in P_{fr} stability (Vince-Prue 1994), but no definite conclusions have been possible to date. However, the ma_3^R mutant of the SDP *Sorghum* is deficient in a B-type phytochrome and is photoperiod insensitive, flowering early under non-inductive (LD) conditions (Pao and Morgan 1986, Childs et al. 1992). This result indicates that in SDP, as in LDP, phyB is required for normal inhibition of flowering under non-inductive conditions. The effect of this mutation on the night-break response has yet to be tested.

Since phyB therefore does not appear to be involved with the night-break response in *Arabidopsis* or the response to a photoperiod extension in pea, the question concerning role of phyB in the control of flowering still remains. In *Arabidopsis* the effects of phyB deficiency on flowering on some genetic backgrounds are expressed under both LD and SD, suggesting that the action of phyB may in fact be relatively unrelated to detection of photoperiod. Instead, it may be a more general inhibitory effect relating to the R:FR ratio of the photoperiod. A similar explanation may be applicable for pea. Mutant *lv* plants abort flower initials and retain many of the vegetative characteristics of a SD-grown WT plant. This phenotype is quite unlike true early day-neutral mutants such as *sn, dne* and *ppd*, which when grown in SD, display the flowering and vegetative phenotype of LD-grown WT plants (e.g. King and Murfet 1985). The effect of *lv* on flower initiation is therefore reminiscent of the effects of a mutation from *LF* to *lf*, which on some backgrounds can cause an early initiating phenotype under SD, but does not alter photoperiod sensitivity in general (Murfet 1985). The case of *lf* demonstrates the way in which allelic variation at a locus essentially unrelated to the detection of photoperiod, may nonetheless interact with the photoperiod detection system and alter flowering behaviour in an apparently photoperiod-dependent manner. The possibility that such a situation also exists for *lv* could be further tested by transfer of *lv* to an inherently later-flowering background. If the early-flowering phenotype of *lv* plants is the result of a threshold effect rather than a direct effect on photoperiod sensitivity, alteration to the genetic background might reveal an effect of *lv* under LD as well as SD.

Role of Phytochrome B in the Control of Phytochrome Reaccumulation

The *lv* mutant shows a clear reduction in the R/FR reversible control of phytochrome reaccumulation. This is in agreement with the previously proposed action of a stable phytochrome in this response (Furuya et al. 1991) and in the control of phyA accumulation generally (Hilton and Thomas 1987). In addition, this result demonstrates clearly the involvement of phyB in the regulation of phyA levels. Further experiments, and use of a

proven phyB-null mutant, will be necessary to determine if phyB acts alone in mediating this response. In any case, this result indicates that the state of phyB at the end of a photoperiod may influence the rate of phyA reaccumulation during a subsequent dark period. This could have important implications for understanding of photoperiodic time measurement since a certain level of phyA is thought to be required for the FR-HIR for promotion of flowering to proceed (Carr-Smith et al. 1994, Vince-Prue 1994).

3.4. Materials and Methods

In general, all plants were grown in either drained, water-saturated vermiculite (experiments in **Figures 3.10, 3.11, 3.13, 3.17**) or in the standard Hobart pea potting mix. Plants used in the photoperiod experiments (**Figures 3.14, 3.15, 3.16**) received an 8 h photoperiod of natural daylight in a heated glasshouse maintained at 23°C, and were transferred into night compartments at 16°C where the photoperiod was extended with incandescent light from Thorn 40W globes at an intensity of ≈3 μmol m^{-2} sec^{-1}.

Standard Hobart light sources were used for all experiments, with the exception of the pulse, fluence rate, R:FR and phytochrome reaccumulation experiments (**Figures 3.11, 3.13, 3.17**) in which standard RIKEN light sources were used. For the R fluence-rate experiments (**Figure 3.11**) the fluence rate of the R source was reduced by filtering through finely perforated metal plates. The WL and supplementary FR sources used in the R:FR experiment (**Figure 3.13**) were identical to those described by López-Juez et al. (1995).

Immunoblotting and *in vivo* spectrophotometric assay for phytochrome were performed as described in **Chapter 2**.

Table 3.1. New designations for *lv* alleles.

Mutant line	Allele	Progenitor cv.	Background
NEU3	*lv-1*	Sparkle	*le a lf sn*
R83	*lv-2*	Sparkle	*le a lf sn*
Wt10895	*lv-3*	Paloma	*le a LF*
L80m	*lv-4*	Hobart L80	*le a st wlo LF*
AF280	*lv-5*	Torsdag	*a LF*
AF300	*lv-6*	Torsdag	*a LF*

Table 3.2. Summary of *lv* segregations.

Allele	Total no.	*LV*	*lv*	$\chi^2_{(3:1)}$	Probability
lv-1	939	705	234	0.65	0.3<P<0.5
lv-2	151	116	35	0.27	0.5<P<0.7
lv-3	337	250	87	0.12	0.7<P<0.9
lv-4	562	406	156	2.28	0.2<P<0.3
lv-5	93	73	20	0.61	0.3<P<0.5
lv-6	28	24	4	1.71	0.2<P<0.3

Table 3.3 F_2 segregation data for *lv* and linkage group VI markers

Loci		Cross[a]	Phenotype[b]						Total	Chi-squared Locus 1	Chi-squared Locus 2	Chi-squared Joint	Linkage Prob.	Recomb. Fraction	SE
			DD	DR	RD	RR									
lv	*wlo*	1	99	2	3	24			128	1.04	1.50	99.4	<0.0001	4.2	1.8
lv	*arg*	1	99	14	13	14			128	1.04	0.67	18.0	<0.0001	26.2	4.7
lv	*pl*	1	87	14	13	14			128	1.04	0.67	18.0	<0.0001	26.2	4.7
wlo	*arg*	1	86	16	14	12			128	1.50	0.67	11.3	<0.001	30.2	5.0
arg	*pl*	1	99	1	1	27			128	0.67	0.67	116.6	<0.0001	1.7	1.1
wlo	*pl*	1	86	16	14	12			128	1.50	0.67	11.3	<0.001	30.2	5.0
lv	*wlo*	2	45	30	24	0			99	0.03	1.48	13.8	<0.001		
lv	*na*	3	130	3	2	46			181	0.22	0.41	156.4	<0.0001	2.7	1.2
			DF	DH	DS	RF	RH	RS							
lv	*prx3*	2	3	32	25	19	3	0	82	0.15	1.98	54.8	<0.0001	7.6	3.0
wlo	*prx3*	2	22	32	6	0	3	19	82	0.15	1.98	44.8	<0.0001	11.0	3.6

[a]Cross: 1) L80m (*lv-4 wlo arg pl*) x L224 (*LV WLO ARG PL*)
2) L232[-] (*lv-1 WLO prx3*[F]) x L111 (*LV wlo prx3*[S])
3) L107 (*LV NA*) x *lv na* segregate from cross NEU3 (*lv NA*) x L81 (*LV na*)

[b]D=dominant, R=recessive, F=homozygous fast, H=heterozygous, S=homozygous slow. The first-named locus is shown first.

Figure 3.1. Phenotype of the *lv-1* mutant. WT ($L232^{+}$) and *lv-1* plants were grown from sowing under short-day conditions (8-h photoperiod of natural daylight under standard phytotron conditions).

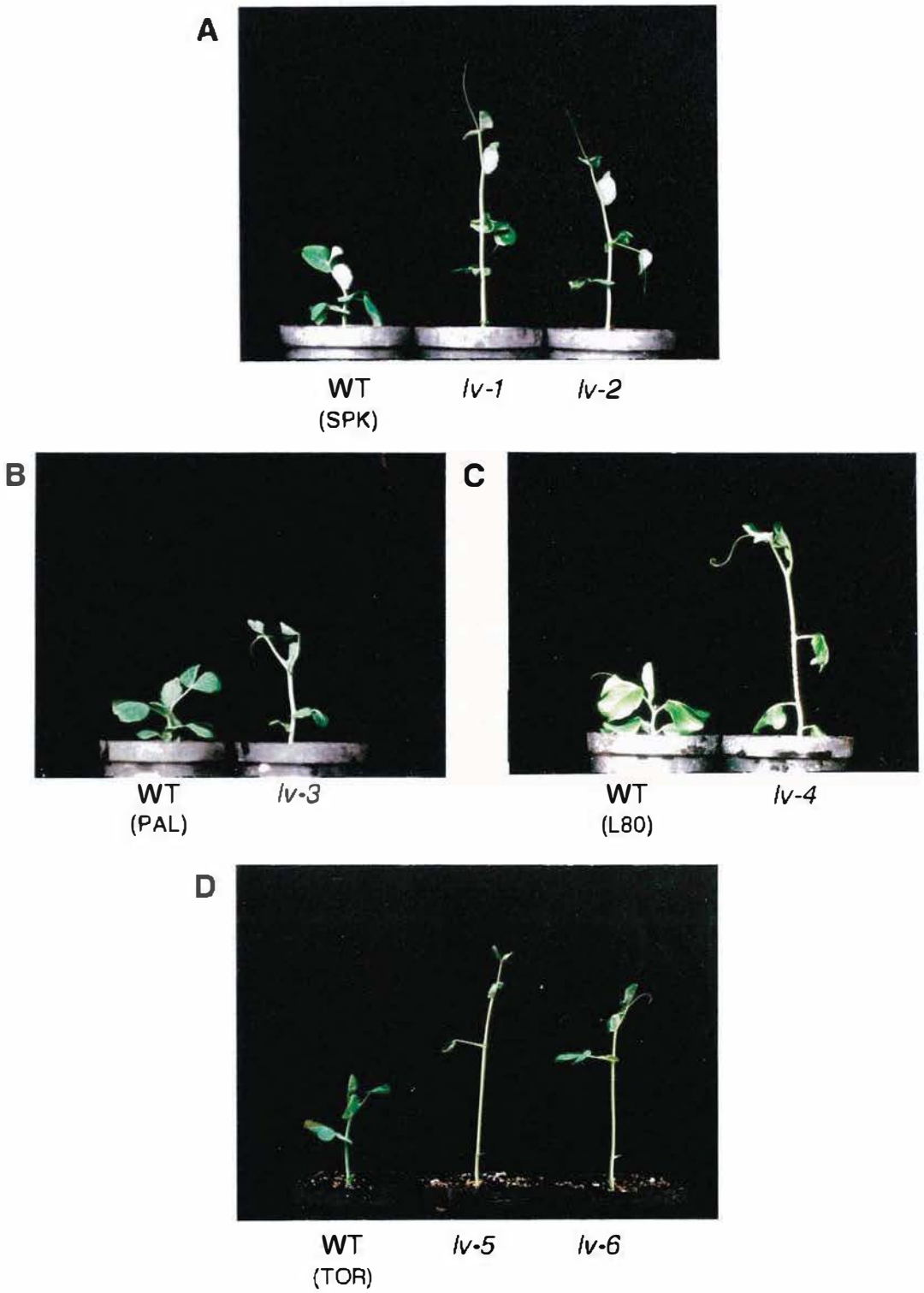

Figure 3.2. Phenotypes of *lv* mutant seedlings grown in white light. Photographs were taken 11 d after sowing. Scale is provided by the pot rim which is 10 cm in diameter. Growing conditions; standard WL, 150 μmol m^{-2} sec^{-1}, 20°C.

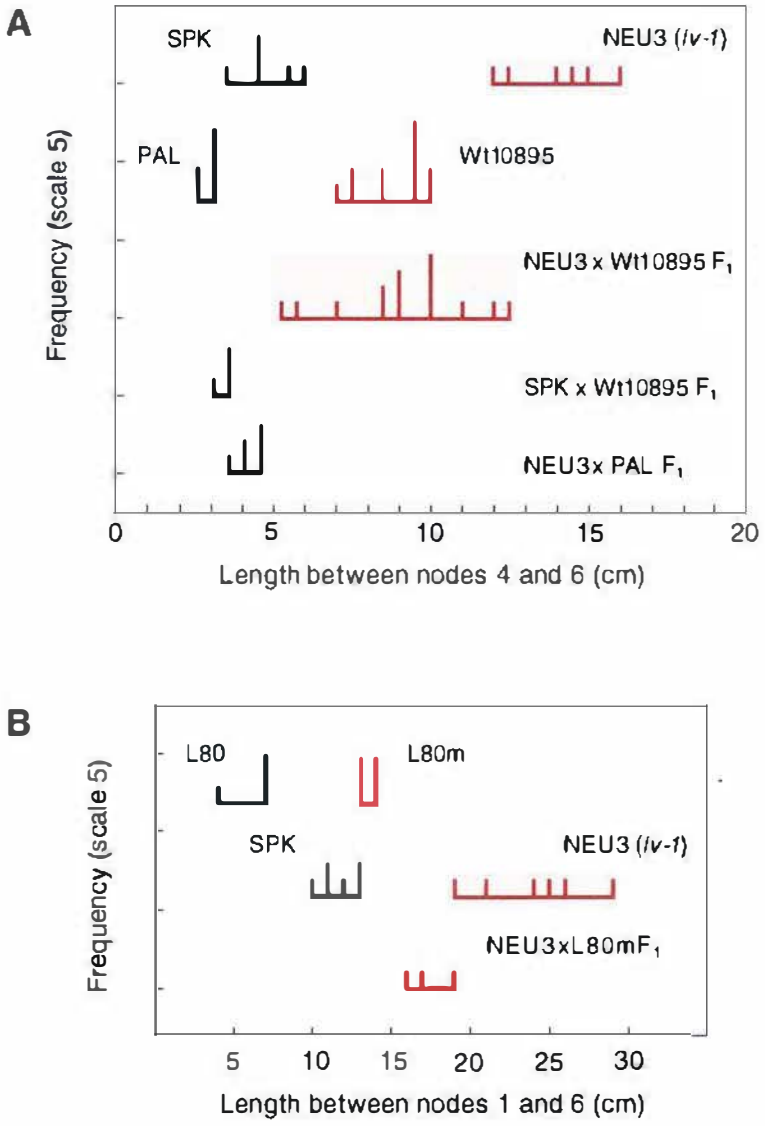

Figure 3.3. Allelism testing of putative *l v* mutant lines Wt10895 (**A**) and L80m (**B**). Allelism with *l v* is confirmed for both mutants by the elongated phenotype of the F_1 in crosses to line NEU3 (*lv-1*). Black and red bars indicate plants with WT and *lv* mutant phenotype, respectively. Growing conditions; standard WL, A. 90 μmol m^{-2} sec^{-1}, 20°C, **B.** 6 μmol m^{-2} sec^{-1}, 17.5•C.

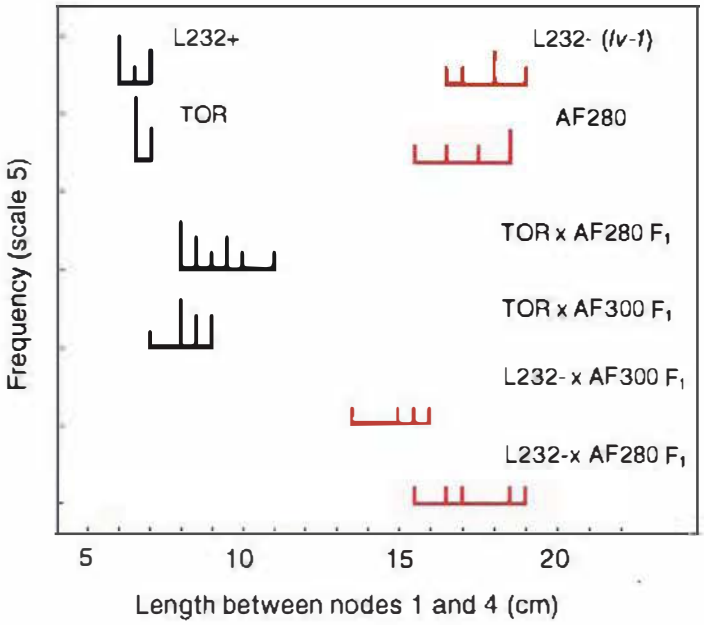

Figure 3.4. Allelism testing of putative *lv* mutant lines AF280 and AF300. Allelism with *lv* is confirmed for both mutants by the elongated phenotype of the F_1 in crosses to L232$^-$ (*lv-1*). Black and red bars indicate plants with WT and *lv* mutant phenotype, respectively. Growing conditions; standard WL, 150 μmol m^{-2} sec^{-1}, 20°C.

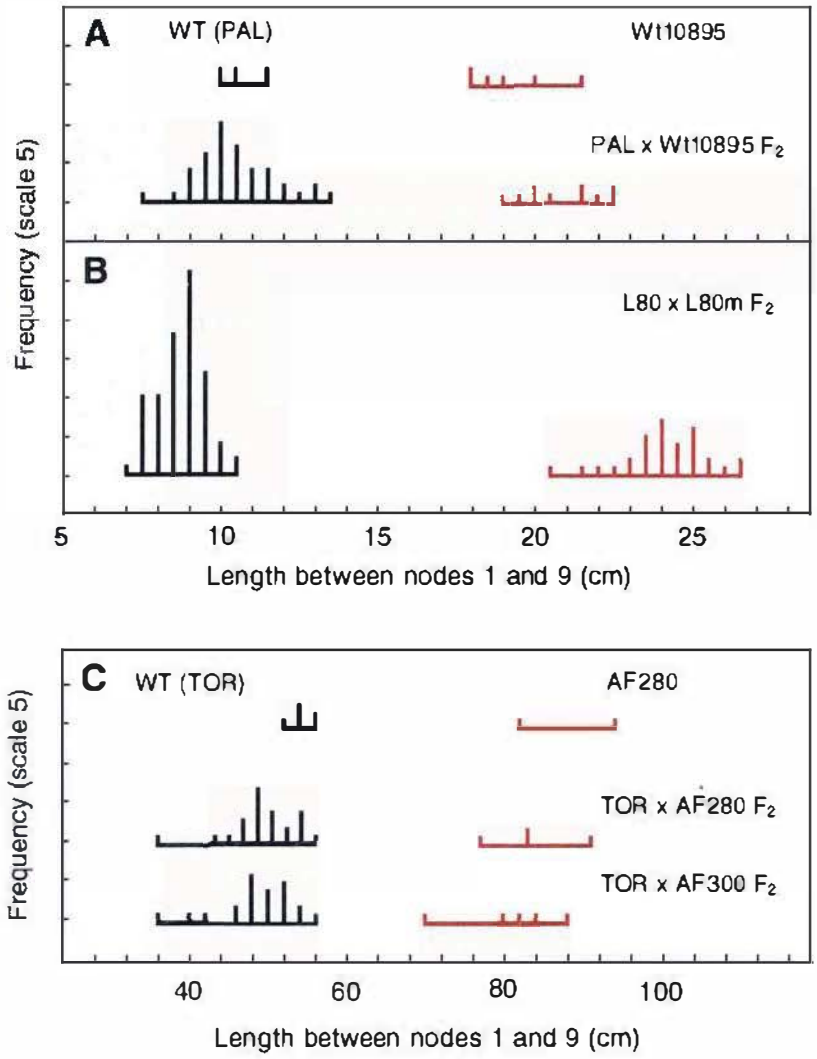

Figure 3.5. Segregation in F_2 populations of crosses between *lv* mutant lines Wt10895 (**A**), L80m (**B**), AF280 and AF300 (C) and their respective progenitor lines. Approximate 3:1 WT:*lv* mutant segregation in each case indicates monogenic recessive inheritance. Black and red bars indicate plants with WT and *lv* mutant phenotype, respectively. Growing conditions (**A**) and (C) 8-h natural light photoperiod under standard phytotron conditions, (**B**) standard WL, 90 μmol m^{-2} sec^{-1}, 20°C.

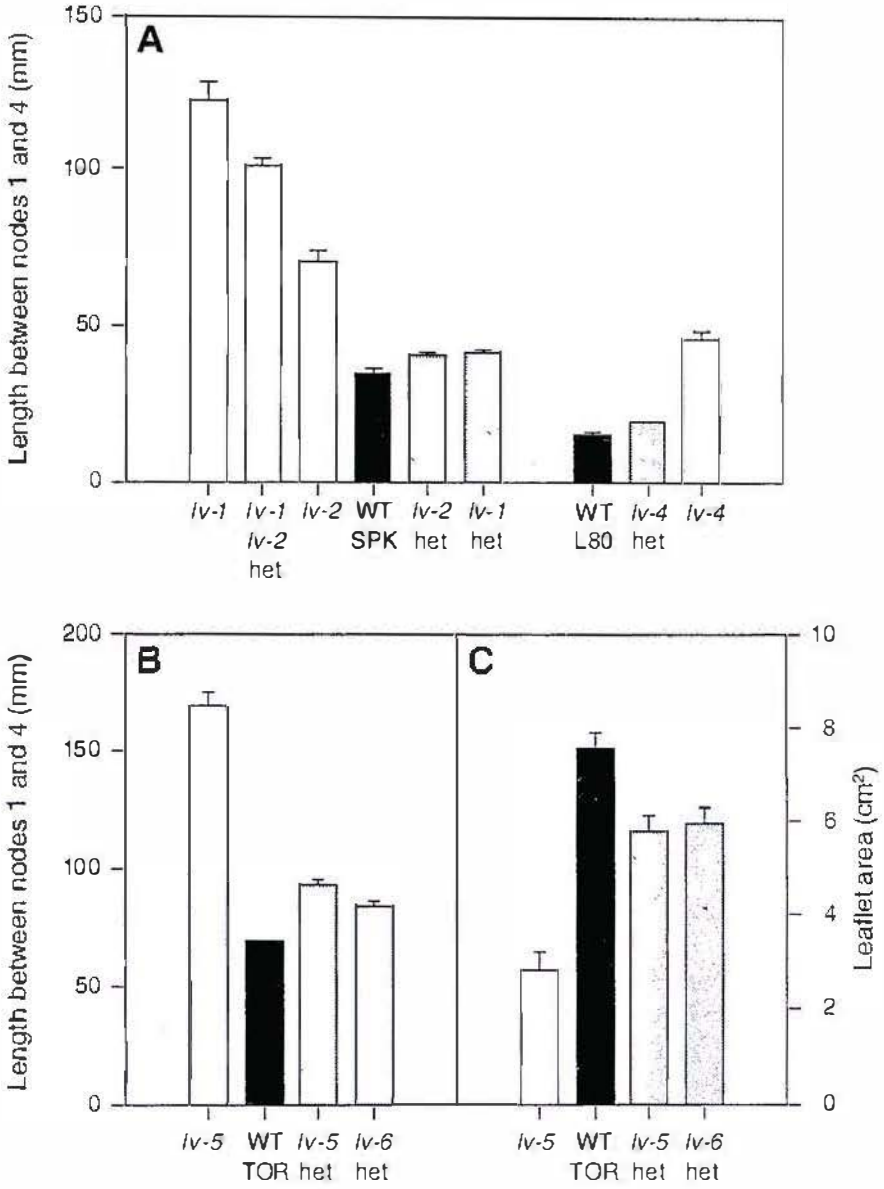

Figure 3.6. Dominance of *LV* over mutant *lv* alleles. Internode length (A,B) and leaf expansion (C) of WT plants, *lv* mutant plants, and heterozygous *LV lv* plants. Plants heterozygous for the various *lv* mutant alleles (*lv-1* het, *lv-2* het, *lv-4* het, *lv-5* het and *lv-6* het) represent F_1 progeny of backcrosses of *lv* mutant lines to their original progenitor lines cv. Sparkle (SPK), cv. Torsdag (T●R) or L80, while *lv-1 lv-2* het indicates the F_1 of a cross between homozygous *lv-1* (NEU3) and *lv-2* (R83) plants. Leaflet area was estimated as the product of the length and width of a single leaflet from the second true foliage leaf (node 4). Growing conditions; standard WL, 150 μmol m^{-2} sec^{-1}, 20°C. Bars indicate SE, n = 10 to 12 (A) or 6-8 (B).

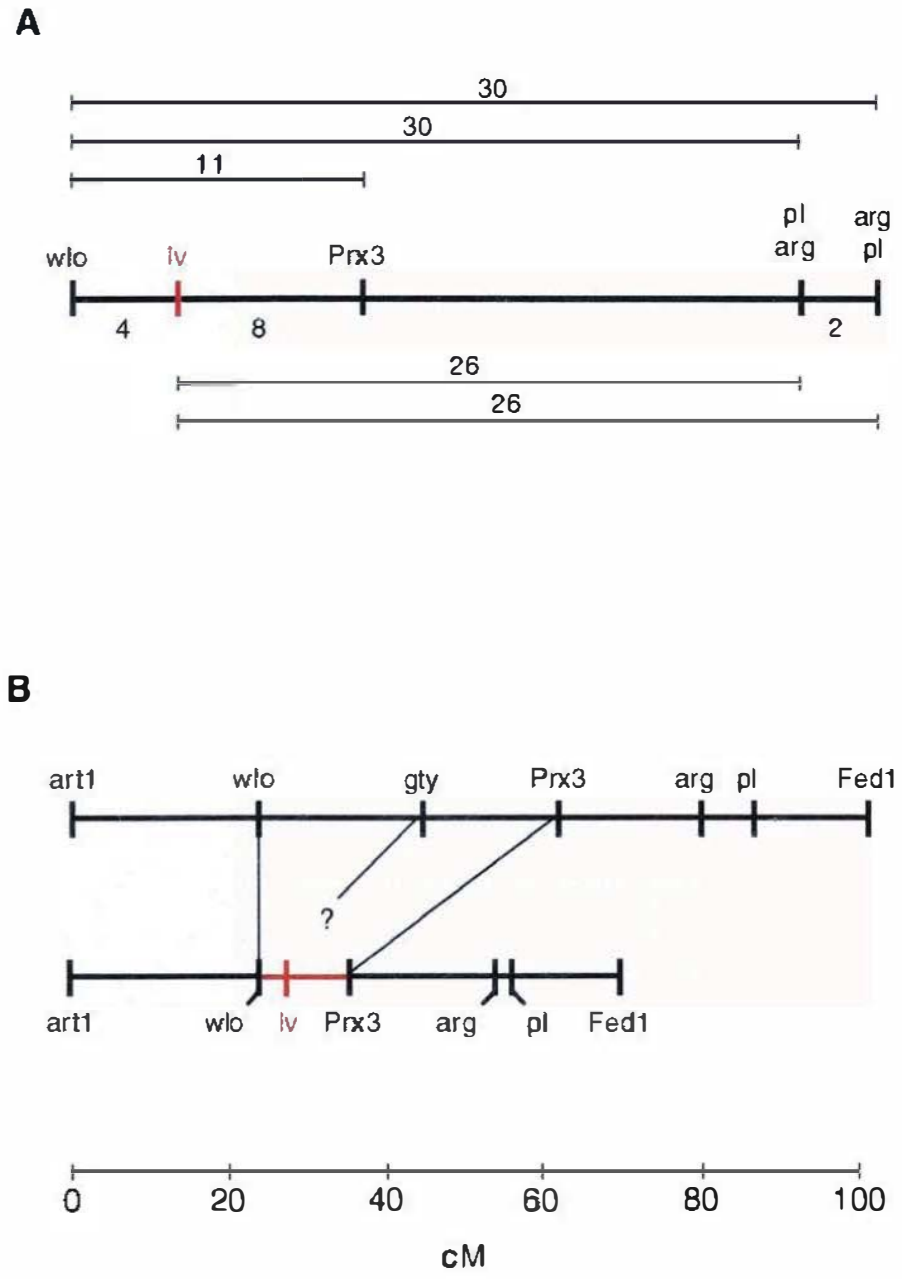

Figure 3.7. Map location of the *lv* gene in linkage group VI. **A.** Partial map constructed from linkage data in Table 2.3 (not to scale). **B.** Complete map of linkage group VI, with the revised *wlo-Prx3* distance shown in red.

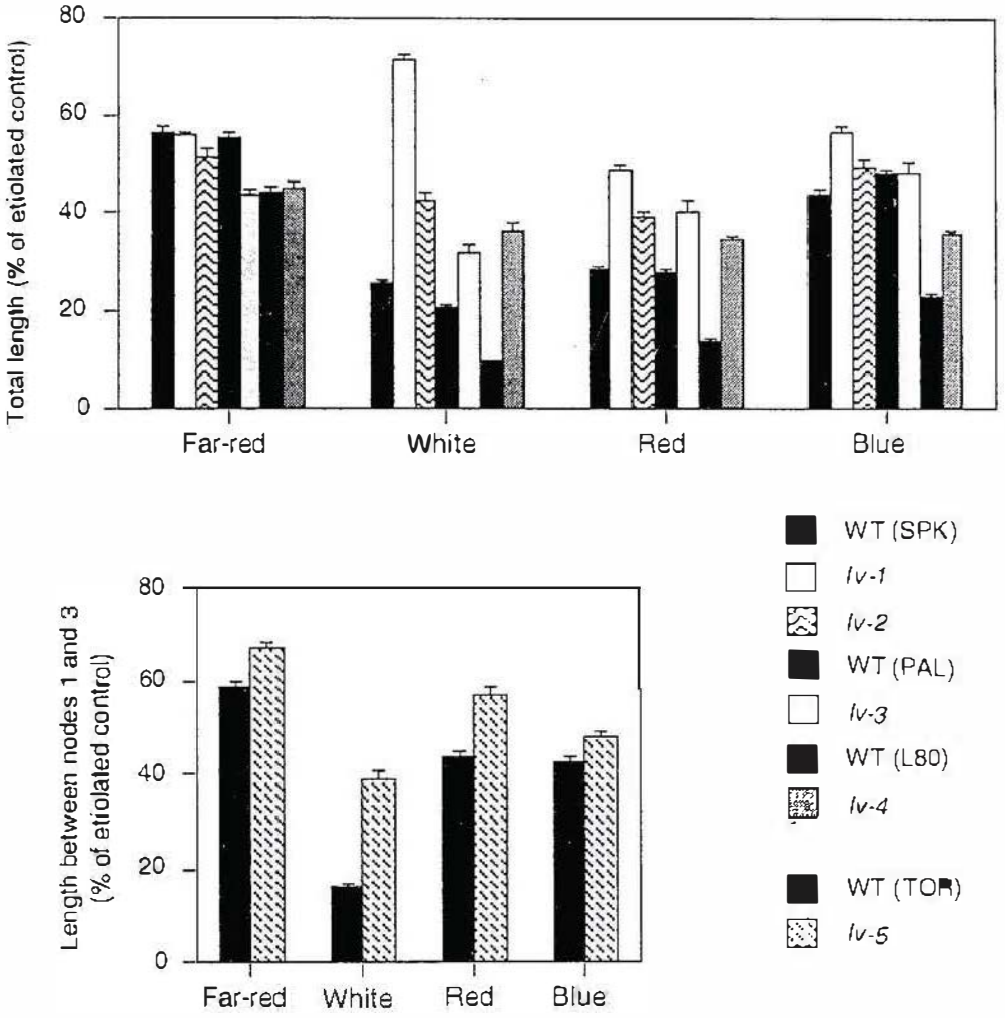

Figure 3.8. Stem elongation of *lv* mutants and corresponding WT lines under continuous monochromatic light. Seedlings were grown in the dark or under FR (8 μmol $m^{-2}sec^{-1}$), R (20 μmol $m^{-2}sec^{-1}$), WL (150 μmol $m^{-2}sec^{-1}$) or B (10 μmol $m^{-2}sec^{-1}$), at 20°C. Total length (top panel) was measured 14 d after sowing. Bars indicate SE, n = 10 to 12.

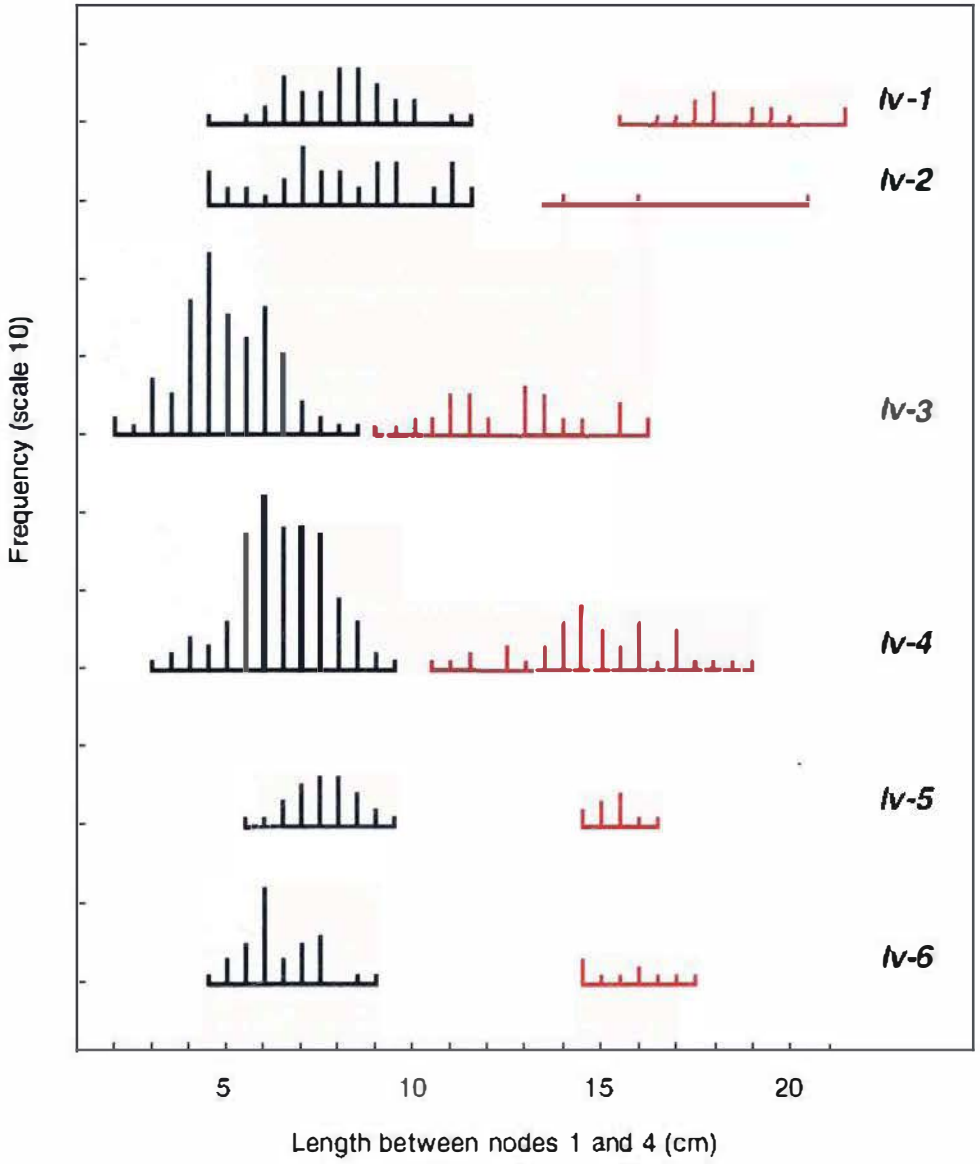

Figure 3.9. Comparison of internode length distributions in the F_2 of back-crosses between *lv* mutant lines and cv. Torsdag. Black and red bars indicate plants with WT and *lv* mutant phenotypes, respectively. Growing conditions; WL, 150 μmol m^{-2} sec^{-1}, 20°C.

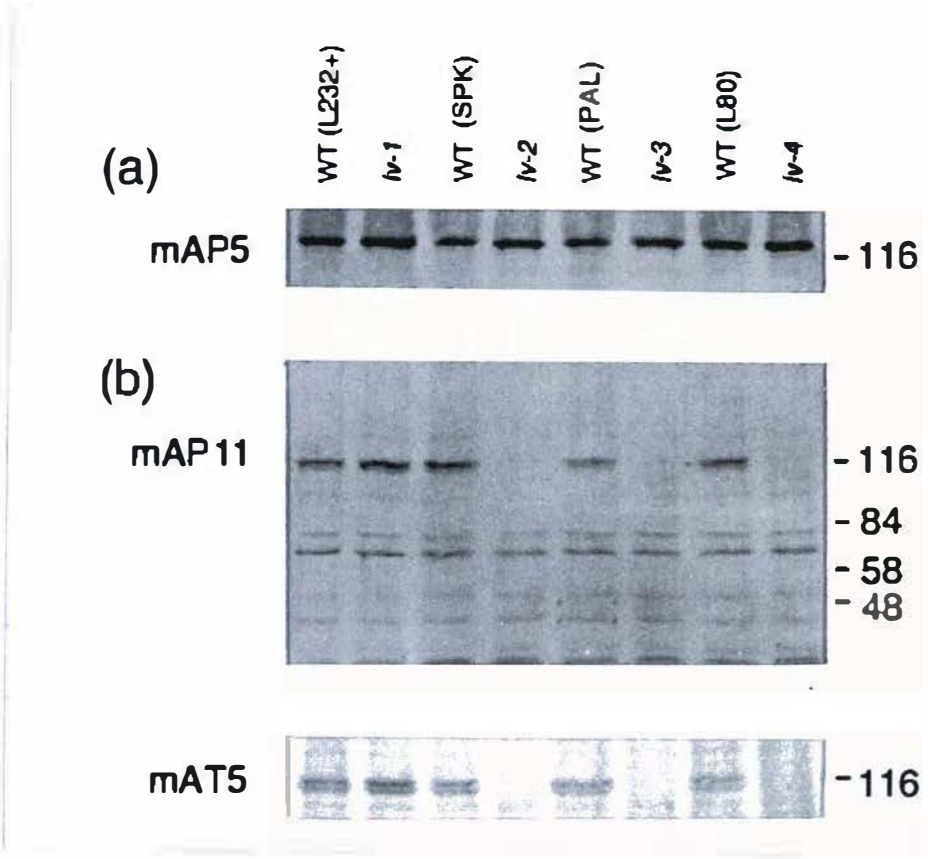

Figure 3.10. Immunoblot detection of PHYA and PHYB apoprotein in crude extracts of etiolated WT and *lv* mutant plants. Each lane contains extract equivalent to 2.5 mg fresh weight. The positions and molecular masses (kD) of prestained markers (Sigma) are indicated at the right of the diagram. (**a**) Detection of phyA by monoclonal antibody mAP5. (**b**) Detection of phyB by monoclonal antibodies mAP11 and mAT5.

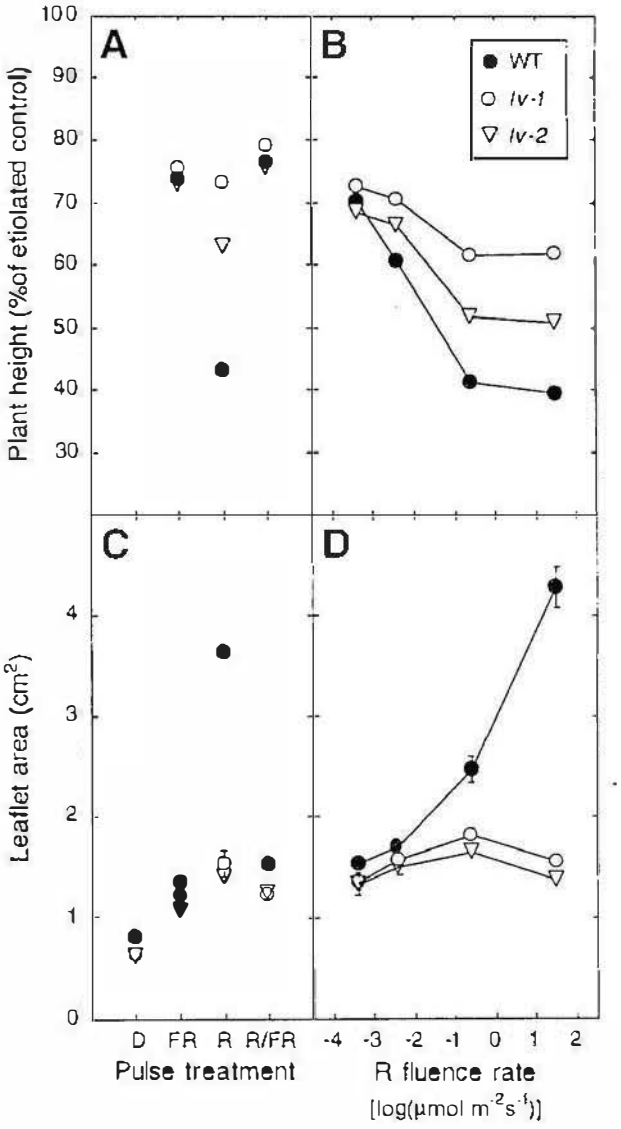

Figure 3.11. Responses to R pulses (A, C) and to fluence rate of continuous R (B, D) in the *lv-1* and *lv-2* mutants. In the pulse experiments, plants were given saturating pulses of R (17 μmol $m^{-2}sec^{-1}$, 10 min), FR (12 μmol m^{-2} sec^{-1}, 15 min), or R followed by FR (R/FR) at 4-h intervals for 10 d after sowing, or maintained in complete darkness (D). Total plant height (A, B) was measured and expressed as a percentage of the height of etiolated plants. Leaflet area (C, D) was estimated as the product of the length and width of a single leaflet from the second true foliage leaf (node 4). Bars indicate SE, n = 12 to 15.

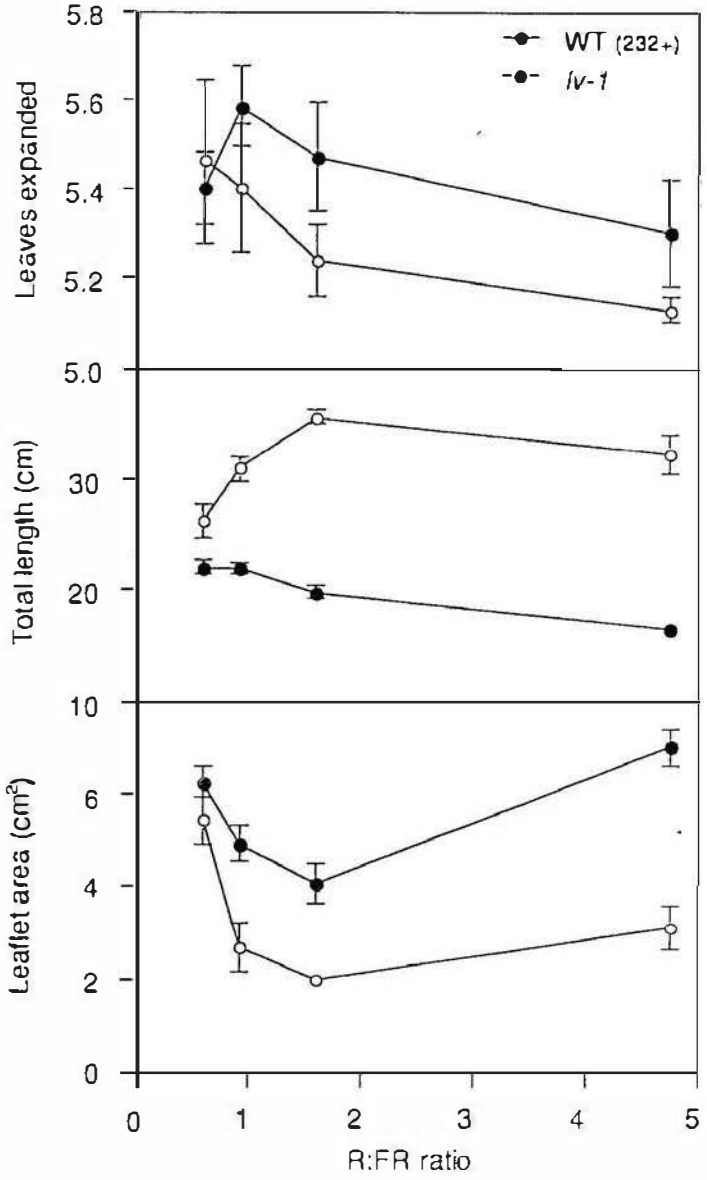

Figure 3.12. Response of *lv-1* plants to R:FR ratio. Plants were grown for 14 d at 20°C in continuous light (60 μmol $m^{-2}sec^{-1}$). Standard WFL was used as the high R:FR source (R:FR = 4.8), and reductions in R:FR were achieved by replacing fluorescent tubes with 60W incandescent globes. Leaflet area was estimated as the product of the length and width of a single leaflet from the second true foliage leaf (node 4). R:FR was calculated as the ratio of fluence rates within the 650-660 nm and 720-730 nm wavebands. Bars indicate SE, n = 10 to 12.

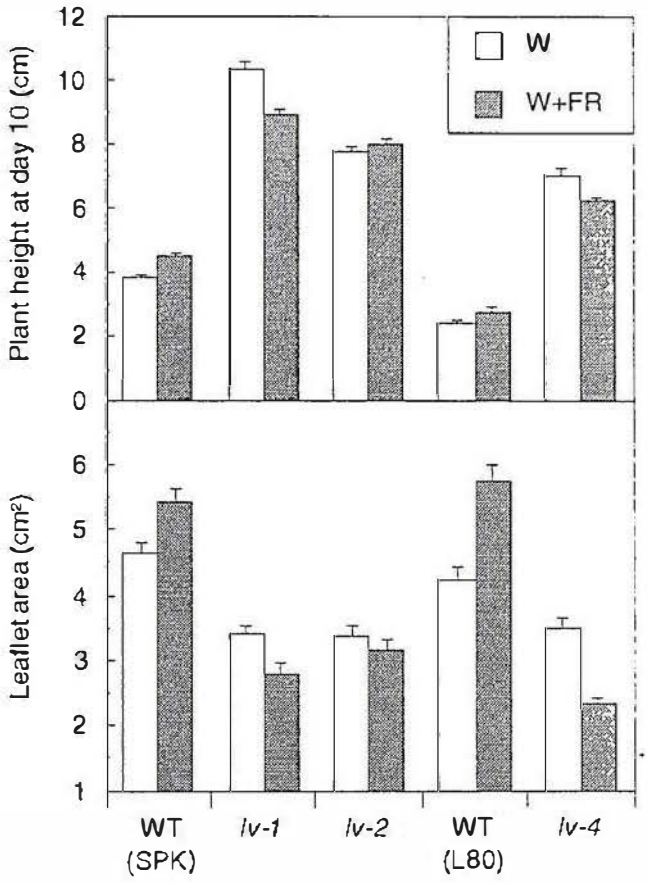

Figure 3.13. Response of *lv-1*, *lv-2* and *lv-4* plants to R:FR ratio. Plants were grown for 10 d at 25°C under continuous WFL (40 μmol m^{-2} sec^{-1}) either with (R:FR = 0.66) or without (R:FR = 5.44) supplementary FR. Leaflet area was estimated as the product of the length and width of a single leaflet from the second true foliage leaf (node 4). R:FR was calculated as the ratio of fluence rates within the 655-665 nm and 725-735 nm wavebands. Bars indicate SE, n = 12 to 15.

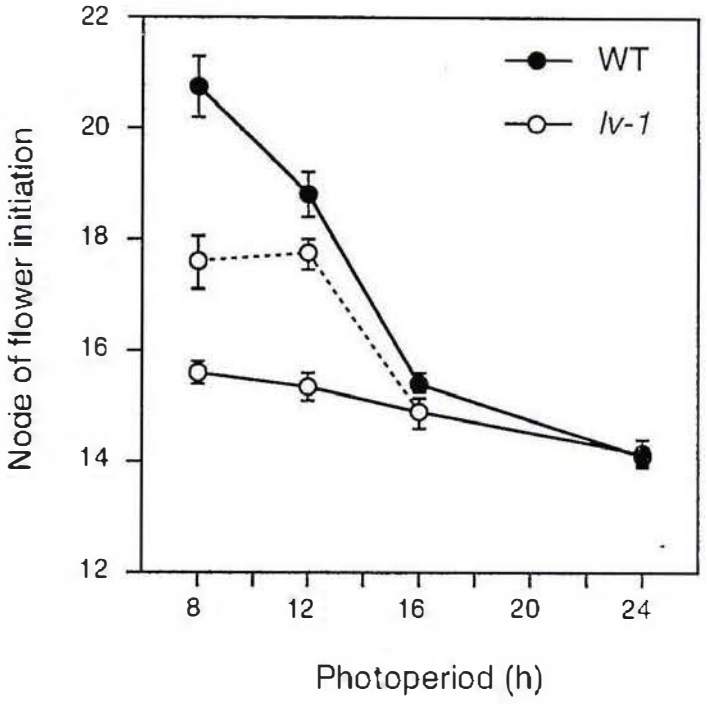

Figure 3.14. Flowering response to photoperiod in the *lv-1* mutant. WT (L232^{+}) and *lv-1* (L232^{-}) plants were grown from sowing in an 8-h photoperiod of natural daylight, extended to 12, 16 or 24 h with weak incandescent light (3 µmol m^{-2} sec^{-1}). The broken line indicates the node of flower development, which differs from the node of flower initiation in *lv-1* plants due to abortion of flower initials. Bars indicate SE, n = 12 to 15

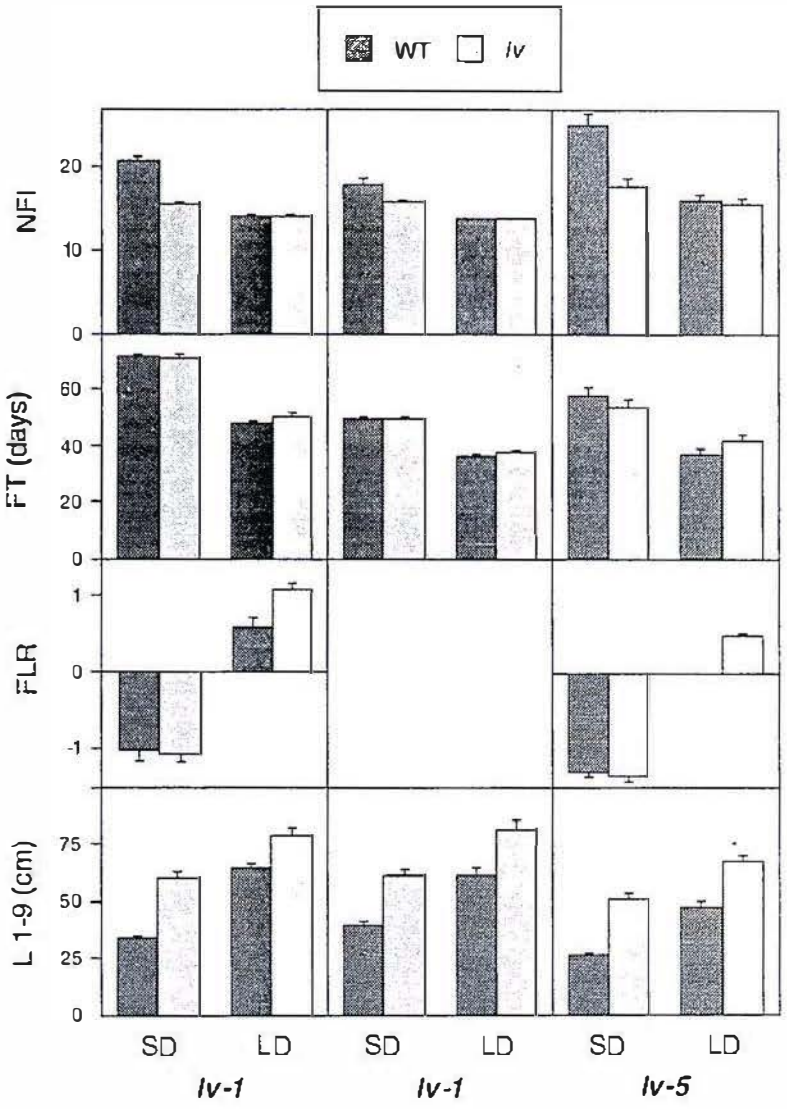

Figure 3.15. Effect of *lv* mutations on responses to photoperiod. The data shown correspond to three separate experiments, two using L232^{-} (*lv-1*) and L232^{+} (WT), and one using AF280 (*lv-5*) and cv. Torsdag (WT). Plants were grown from sowing in an 8-h photoperiod of natural daylight with or without a 16-h extension given as weak incandescent light (3 μmol m^{-2} sec^{-1}). NFI - node of flower initiation, FT - time to first open flower, FLR - flower/leaf relativity index, L 1-9- stem length between nodes 1 and 9. Bars indicate SE, n = 8 to 12.

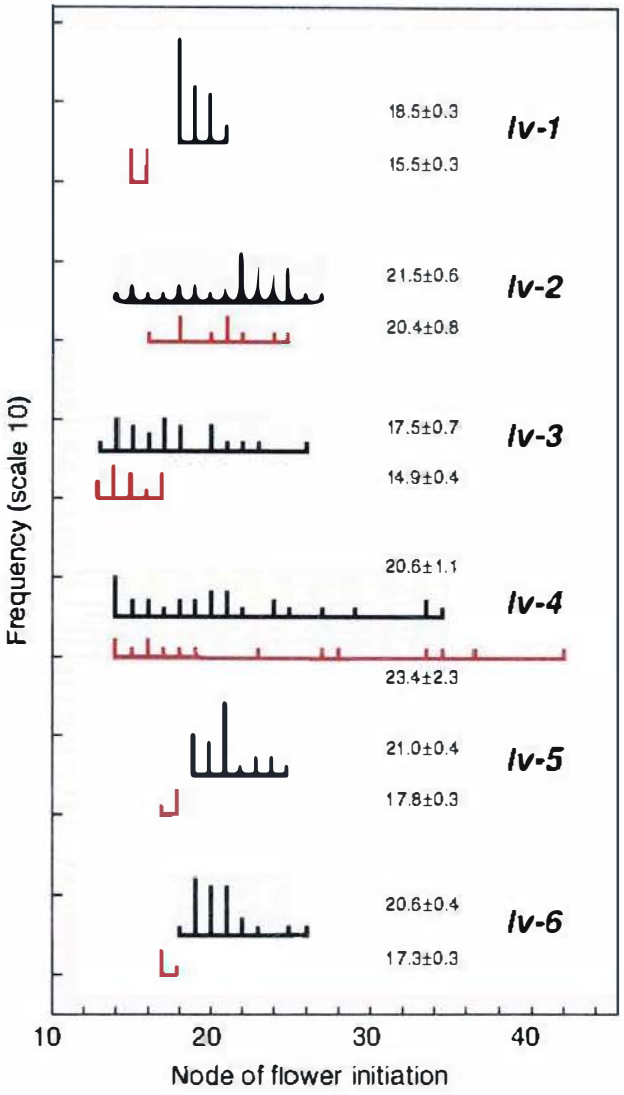

Figure 3.16. Comparison of distributions for node of flower initiation in the F_2 of back-crosses of *lv* mutant alleles to cv. Torsdag. Original *lv* mutant lines (see Table 3.1) were used as the parental line in all crosses, except for the cross involving *lv-1*, in which L232 was used. In some crosses, the *le* and/or *sn* mutations were also segregating, but only tall (*Le*) photoperiodic (*Sn*) segregates are shown here. Plants were grown from sowing under short-day conditions (8-h photoperiod of natural daylight). Black and red bars indicate plants with WT and *lv* mutant phenotype, respectively.

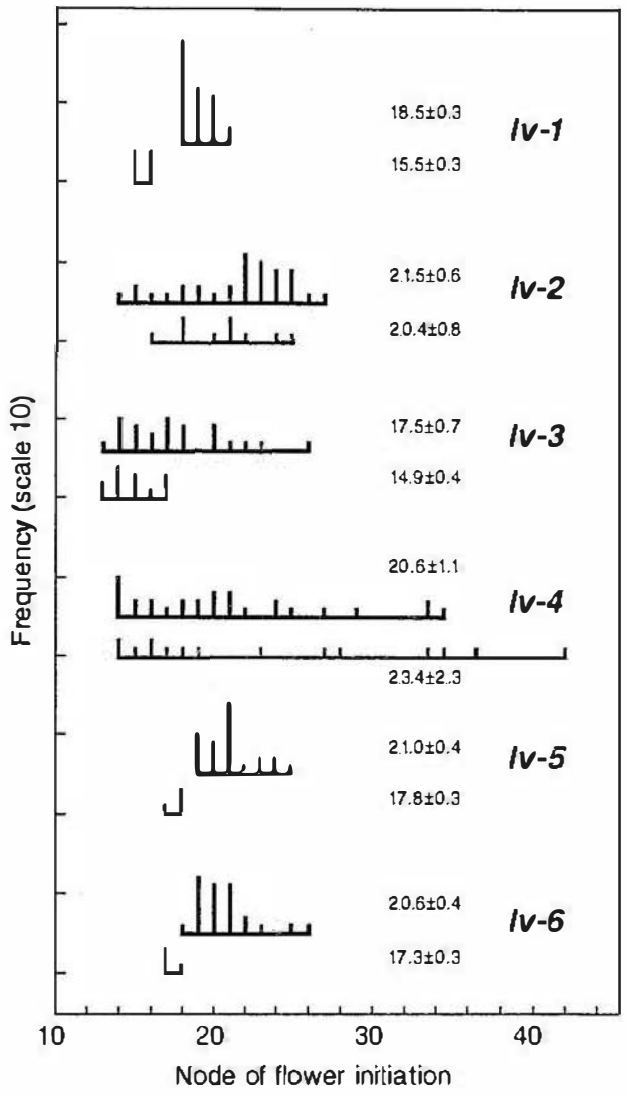

Figure 3.16. Comparison of distributions for node of flower initiation in the F_2 of back-crosses of *lv* mutant alleles to cv. Torsdag. Original *lv* mutant lines (see Table 3.1) were used as the parental line in all crosses, except for the cross involving *lv-1*, in which L232 was used. In some crosses, the *le* and/or *sn* mutations were also segregating, but only tall (*Le*) photoperiodic (*Sn*) segregates are shown here. Plants were grown from sowing under short-day conditions (8-h photoperiod of natural daylight). Black and red bars indicate plants with WT and *lv* mutant phenotype, respectively.

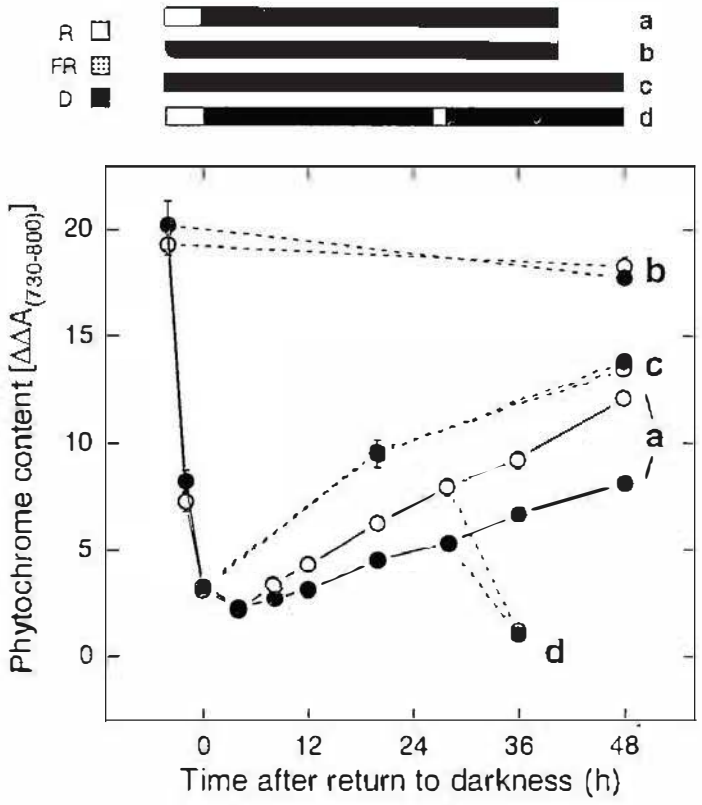

Figure 3.17. Destruction and reaccumulation kinetics of spectrophotometrically detectable phytochrome in WT (closed circles) and *lv-4* mutant plants (open circles). Plants were grown for 5 d in complete darkness and then subjected to light treatments. (a) 4 h R (17 μmol m^{-2} sec^{-1}) followed by return to darkness; (b) maintained in continuous dark; (c) 4 h R, 15 min FR (12 μmol m^{-2} sec^{-1}), then returned to darkness; (d) 4 h R, 28 h dark, 1 h R, 7 h dark. These light treatments are indicated diagrammatically at the top of the figure. Each point for the basic reaccumulation timecourse (a) is the mean of at least four replicates (from separate plantings) with three determinations per replicate, while all other points are the mean of two such replicates. Bars indicate SE; where not visible, bars are smaller than the plot symbols.

4. Mutants Deficient in Phytochrome A: The *fun1* Locus

4.1. Introduction

The isolation of pea mutants deficient in phytochrome B has demonstrated an important role for this phytochrome in the mediation of responses to R in pea seedlings (**Chapter 3**). However, the retention of various responses to both R and FR in phyB-deficient *lv* plants indicates that other phytochromes must be active in the control of photomorphogenesis in both seedlings and mature plants. In particular, the normal response of etiolated *lv* seedlings to continuous FR (the FR-HIR) indicates that a photoreceptor other than phyB is responsible for FR perception.

Several lines of circumstantial evidence have linked the FR-HIR with phyA, the light-labile phytochrome abundant in etiolated tissue. Firstly, seedlings apparently lose the FR-HIR upon deetiolation, and this loss is associated with a decline in the levels of phyA (Beggs et al. 1980, Holmes and Schäfer, 1981). Secondly, overexpression of phyA leads to an enhanced sensitivity to FR (McCormac et al. 1991, 1993). Thirdly, mutants which are deficient in spectrophotometrically detectable phytochrome (predominantly phyA), such as *au* in tomato and *hy1* in Arabidopsis, are also insensitive to continuous FR (Koornneef et al. 1980, 1985). Recent identification of phyA-deficient mutants in *Arabidopsis* and tomato have confirmed that the FR-HIR is in fact controlled by phyA (Parks and Quail 1993, Nagatani et al. 1993, Whitelam et al. 1993, Dehesh et al. 1993, van Tuinen et al. 1995a). These mutants have also demonstrated a role for phyA in the control of responses to FR-rich light in the mature plant, such as the flowering response to daylength extension (Whitelam et al. 1993, Halliday et al. 1994). Therefore, as the next step in the dissection of phytochrome control of photomorphogenesis in pea, I considered it important to isolate a phyA-deficient mutant of pea. To this end, an EMS-mutagenised population was screened for mutants showing reduced response to FR. This chapter reports on the isolation and characterisation of mutants specifically deficient in phyA.

4.2. Results

4.2.1. Isolation of FR-Unresponsive Mutants

Approximately 6000 M_2 seedlings representing 1100 M_1 families obtained from EMS mutagenesis of cv. Torsdag, were screened under continuous FR light (8 μmol m^{-2} sec^{-1}). WT pea plants grown under these conditions show decreased internode length, increased leaflet expansion, and an increased rate of node production relative to plants grown in darkness (see **Figure 1.1B**). Four mutants were isolated which failed to respond to the FR, exhibiting the long internodes, unexpanded leaves and apical hook typical of pea seedlings grown in complete darkness (**Figure 4.1A**). One of these mutants was lethal, failing to de-etiolate on transfer to natural daylight. This mutant was not recovered from progeny of sibling plants.

In *Arabidopsis*, insensitivity to continuous FR has been shown to result either from a specific deficiency in phyA (Parks and Quail 1993), or from a deficiency in the phytochrome chromophore (Koornneef et al. 1980, Parks and Quail 1991). Unlike phyA-deficient mutants however, chromophore-deficient mutants are also insensitive to R (Koornneef et al. 1980). Therefore, in order to distinguish potential chromophore-deficient mutants from phyA-specific mutants the three remaining FR-insensitive lines were screened under continuous R in the M_3 generation. One of the lines (AF130) was found to have a dramatically reduced response to R as well as to FR, and characterisation of this mutant is presented in **Chapter 6**. The two remaining lines (AF140 and AF188) had normal responses to R and were retained as possible candidates for specific phyA deficiency. The phenotype of the AF140 mutant under FR is shown in **Figure 4.1B.**

4.2.2. Inheritance of the AF140 Mutant Phenotype

Complementation testing showed the AF140 and AF188 mutants to be allelic (**Figure 4.2**), and AF140 was selected for further investigation. Grown under FR, the F_2 generation of the backcross of AF140 to its parental line cv. Torsdag segregated into two classes (**Figure 4.2B**) in close accord with a 3:1 ratio (39:11, $\chi^2_{(3:1)}=0.240$, $0.5<P<0.7$). The AF140 phenotype therefore results from a recessive mutation at a single locus, which was designated *f*ar-red *un*responsive 1 (*fun1*). Similar results were obtained in the F_2 of the cross TOR x AF188 (**Figure 4.2B**). The mutant alleles in lines AF140 and AF188 were designated *fun1-1* and *fun1-2*, respectively. In addition to the lack of response to FR, mature *fun1-1* and *fun1-2* plants grown under an 18-h photoperiod in the glasshouse showed a striking phenotype characterised by dwarfing and delayed flowering and senescence (**Figure 4.3**), which

greatly facilitated selection of mutant plants in subsequent genetic analyses. In a separate F_2 generation of the cross TOR x AF140 in which plants were grown to maturity, the reduced internode length and delayed senescence seen in *fun1-1* plants showed perfect co-segregation indicating that these are pleiotropic effects of the *fun1* mutation (**Figure 4.4**). A similar segregation was again obtained in the cross TOR x AF188 (**Figure 4.4**).

4.2.3. Map location of *fun1*

To determine the linkage relationships of the *fun1* locus, joint segregation of *fun1* and various morphological marker loci was scored in the cross AF140 x L111 (Marx's multi-marker line A875-55-0). All individual segregations were in accord with expectation ($P>0.2$). Significant linkage was detected between *fun1* and *a* in linkage group I and between *fun1* and group II markers *k* and *wb* (**Table 4.1**). This evidence is relatively strong ($P<10^{-4}$ for each joint segregation) and confirms recent findings that the bottom end of the top section of group I (as shown in the map of Weeden et al. 1993) is joined to the bottom end of group II (Paruvangada et al. 1995, N Ellis pers. comm., IC Murfet pers. comm.). These results place *fun1* 27 cM from *k* and approximately 39 cM from *wb*. The distance between *fun1* and *a* of about 34 cM therefore implies a distance of about 60 cM between *k* and *a*, which suggests that that the bottom of the top half of group I and the bottom of group II not only join but also overlap (**Figure 4.5**). Several loci have recently been mapped below *a* on group I, including *His7* (Kosterin 1992), *blb* (Kosterin and Rozov 1993), *waxy1* (Kovalenko and Ezhova 1992) and *Enod2* (Paruvangada et al. 1995). The most distant of these loci, *blb*, is approximately 34 cM below *a* (Kosterin and Rozov 1993, IC Murfet, pers. comm.), and the present results thus predict *blb* and *fun* to be linked within about 5 cM (**Figure 4.5**). Further examination of linkage relationships will also be required to determine the positions of "group II" loci *Fum* and *Dia3* relative to *fun1*, *blb*, *Enod2* and "group I" loci *His 7*, *lf* and *a* (**Figure 4.5**).

4.2.4. Spectral Sensitivity

Mutant *fun1-1* plants grown in FR did not differ significantly from etiolated wild type plants with respect to either internode length or leaf expansion (**Figure 4.2A**) indicating that the *fun1-1* mutant is completely lacking in the ability to respond to FR. In fact, *fun1-1* plants grown in FR were occasionally observed to be slightly more etiolated than dark-grown WT plants (e.g. see **Figure 4.8**), although this was probably due to slight de-etiolation of the WT in response to brief exposure with dim green safelight. In contrast, *fun1-1* seedlings grown under R or WL were difficult to distinguish from WT (**Figure 4.6**), and under B, *fun1-1* seedlings were generally slightly shorter than WT (**Figure 4.6A**). A

specific insensitivity to FR and the lack of a clear phenotype in WL are characteristic of mutants in *Arabidopsis* and tomato which are deficient in functional phytochrome A (Van Tuinen et al 1995a, Parks and Quail 1993, Whitelam et al 1993).

4.2.5. Phytochrome Levels

To test whether phytochrome A was selectively affected in the *fun1-1* mutant, phyA and phyB apoprotein levels were examined by immunoblotting. **Figure 4.7** shows clearly that PHYA is undetectable in crude protein extracts from etiolated *fun1-1* seedlings. In contrast, the PHYB detected by monoclonal antibody mAT5, which is lacking in *lv* mutants (**Figure 3.10**), is present at normal level in *fun1-1*.

An examination of phytochrome levels in etiolated *fun1-1* seedlings by *in vivo* difference spectrophotometry revealed the *fun1-1* mutant to possess only about 2.5% of the photoreversible phytochrome present in the wild type (**Table 4.2**). This indicates that phyA accounts for at least 97.5% of the spectrally active phytochrome in etiolated pea seedlings. After 4 h R treatment, phytochrome in WT seedlings was depleted to about 15% of the dark level (**Table 4.2**) while the phytochrome level in *fun1-1* seedlings remained similar (about 2.5%). On the basis of the results in **Table 4.2**, phyA therefore makes up about 80% of the phytochrome pool present after 4 h R. Although this almost certainly overestimates the proportion of phyA in the stable pool, since phytochrome in WT seedlings can be depleted below the level present after 4 h R (see **Figures 3.17, 7.11**), it does indicate the presence of a substantial amount of phyA in the stable pool, which is in keeping with the obvious effects of phyA deficiency on the mature plant phenotype (**Figure 4.3**). The small pool of photoreversible phytochrome remaining in *fun1* plants (about 2.5% of etiolated WT level) must therefore consist mostly of other, light-stable phytochromes. Effects of the phytochrome B-deficient *lv-5* mutant on the level of spectrally active phytochrome are undetectable (**Table 4.2**), suggesting that the contribution from phyB is small, and implying the presence of a third phytochrome. However, this may be difficult to demonstrate by *in vivo* spectrophotometry, since the phytochrome content in standard samples of *fun1* tissue is close to the detection limit of the system. Also, leakiness of the *fun1-1* and *lv-5* mutations can not currently be ruled out.

4.2.6. R/FR Reversibility of De-Etiolation in the *fun1* Mutant

It was shown in **Chapter 3** that the de-etiolation response of pea seedlings to intermittent R pulses consists of two components; a FR-reversible component which is lacking in phyB-deficient *lv* mutants and is therefore mediated by phyB, and a non-FR-reversible

component which is retained in phyB-deficient mutants. Since this latter component is essentially an inductive response to FR, it was suggested that it might be controlled by phyA. **Figure 4.8** shows that the *fun1-1* mutant responds to R pulses slightly less strongly than WT, and that the response of the mutant retains a similar degree of FR-reversibility to WT. However, the response to terminal FR pulses is substantially reduced in the *fun1-1* mutant, indicating that this component is controlled by phyA. This result also suggests that phyA and phyB together control most if not all of the response to intermittent R pulses. PhyB-deficient pea mutants also retain a normal response to continuous R at fluence rates below about 4×10^{-4} μmol m^{-2} sec^{-1}, and it is likely that this response is also controlled by phyA, although this has yet to be directly tested.

4.2.7. Phenotype of Mature *fun1* Plants

Mutant *fun1* seedlings were virtually indistinguishable from WT when grown for up to 12 d under continuous cool-white fluorescent light (**Figure 4.6B**). However, as mentioned above, mature *fun1* plants grown under standard glasshouse LD conditions exhibited a striking phenotype (**Figure 4.3**), with a reduction in internode length of up to 50% relative to WT (**Table 4.3**). The onset of this dwarf phenotype was monitored in more detail by measuring lengths of individual internodes in *fun1* plants grown in the glasshouse. Under these conditions, internodes in the *fun1* mutant were slightly longer than WT until about node 6, beyond which a dwarfing effect of *fun1* gradually became apparent (**Figure 4.9B**). The reduction in internode length in *fun1* plants coincided with a thickening (**Figure 4.9A**) and paling of the stem, which in later internodes often took on a yellowish, succulent appearance, and showed prominent transverse banding (**Figure 4.9C**). This commenced at about node 10 and became particularly severe several internodes below the node of flower intiation. The severity of the stem phenotype appeared to gradually diminish once regular flower development was established, and above about node 24 the stems of *fun1* plants regained a relatively normal appearance.

Whereas WT plants do not show any lateral branching when grown under standard glasshouse LD conditions, *fun1* plants branched strongly both from basal and aerial nodes (**Figure 4.10**). Under these conditions WT plants flower at about node 16. Flowers develop on relatively short peduncles (**Table 4.3**) and set strongly, with plants producing 5-6 flowering nodes and 20-30 seeds before apical arrest (**Table 4.3**). However, in *fun1* plants, flower initials did not appear until about node 19 and did not develop until about node 24 (**Table 4.3, Figure 4.9C**), with the first developed flower opening much further behind the apical bud (lower FLR) and on a much longer peduncle than in WT plants (**Table 4.3**). Perhaps most striking was the large delay in senescence of *fun1* plants, which produced

four times as many reproductive nodes before senescence and gave two to three times the yield (60-100 seed) of WT plants (**Table 4.3**). In some cases, *fun1* plants grew for more than six months before senescence, where WT plants senesced and dried after about 3 months. The *fun1* mutation also appeared to alter leaf development, conferring a characteristic buckled appearance to leaflets (**Figure 4.11A**) suggestive of a greater rate of expansion of interveinal photosynthetic tissue relative to vascular tissue. Finally, *fun1* leaves developed to a greater degree of complexity, with some leaves developing 4 pairs of leaflets and thus exceeding the maximum of 3 pairs seen in WT plants (**Figure 4.11B**).

4.2.7. Photoperiod Responses in the *fun1* Mutant

The phenotypic syndrome exhibited by *fun1* mutant plants in LD is very similar to that shown by WT plants grown in SD, which also show delayed flower initiation, retarded flower and fruit development, longer peduncles, delayed transition to more complex leaf pattern, and delayed onset of apical senescence (Murfet 1982). I therefore considered that *fun1* plants might be unable to detect the difference between long and short day conditions. To test this, WT and *fun1-1* plants were grown under SD (8 h) and standard phytotron LD conditions (8 h extended with 16 h weak incandescent light). A photoperiod extension with light establishing an intermediate photoequilibrium has previously been found to be the most effective for the promotion of flowering in pea (Reid and Murfet 1977) and various other LDP species (e.g. Evans 1976, Downs and Thomas 1982, Carr-Smith et al. 1989), while the low fluence rate used in the extension excludes a significant contribution to total PAR.

Figure 4.12 shows that *fun1* plants are in fact very similar in appearance to WT plants grown in SD. WT plants elongate and flower earlier in response to a photoperiod extension, whereas *fun1* plants are essentially unresponsive to the extension, showing the same growth habit in LD and SD. The data in **Figure 4.13** confirm the lack of response of *fun1* plants to the photoperiod extension and show that it is manifest in a number of different characters, including time to first open flower, and number of reproductive nodes. These results indicate that phyA is the primary phytochrome responsible for the detection of a FR-rich photoperiod extension in pea.

Since *fun1* seedlings are specifically insensitive to FR, it was considered that the inability of *fun1* plants to detect a photoperiod extension might be dependent on the spectral quality of that extension. However, *fun1* plants were similarly unresponsive to 16-h extensions with weak fluorescent light, which contained essentially no FR (**Figure 4.14**). In fact, a small promotive effect of the incandescent relative to the fluorescent

extension was observed. At least in terms of stem elongation, it is possible that this difference might reflect an EOD-FR response mediated by phyB, since it is eliminated by the *lv-1* mutation (**Figure 4.14**). Regardless of this small difference, it appears that in pea, phyA is active in the detection of low-fluence-rate photoperiod extensions at both high and low R:FR, and that under neither regime are other phytochromes able to compensate for the loss of phyA. The apparent FR specificity seen for phyA-induced de-etiolation in pea seedlings therefore does not necessarily exist for other photoresponses.

4.2.8 Isolation of a *fun1 lv* Double Mutant

In order to further define the roles of phytochromes A and B, a *fun1 lv* double mutant was isolated in the F_2 of the cross AF140 (*fun1-1*) x AF280 (*lv-5*). Initially, the *lv* segregates in this cross were identified by their increased elongation and reduced leaf expansion under WL. Several slightly shorter *lv* segregates were identified as putative double mutants. Plants were then transferred to the glasshouse apron where the double mutants were more clearly distinguished. Identity of double mutants was confirmed by selection from *lv* families segregating *fun1* in the M_3 generation, and by backcrossing.

Grown under standard WL conditions, double mutant plants showed the characteristic appearance of the *lv* single mutant, with pale elongated internodes and reduced leaf development, but were slightly shorter and had poorer leaf development than the single *lv* mutant (**Figure 4.15A**). The difference between *lv* and *fun1 lv* plants became more pronounced after transfer of plants to the glasshouse where they received a natural photoperiod extended to 18 h with mixed fluorescent/incandescent light (**Figure 4.15B**). Under these conditions, the phenotype of the *fun1 lv* double mutant was strikingly different from WT and from either single mutant. Both *fun1* and *lv* single mutants grown in the glasshouse achieve essentially full de-etiolation, whereas the double mutant retained an appearance reminiscent of dark-grown plants, with pale stem, elongated petioles and poor leaflet development. Although the presence of the *lv* mutation somewhat alleviated the dwarfing effect of *fun1* on internodes, *fun1 lv* double mutant internodes showed a paling and thickening considerably more severe than the *fun1* single mutant (**Figure 4.16**). For several nodes below NFI, the internodes of *fun1 lv* plants appeared totally lacking in chlorophyll and had a fattened, distorted appearance which in more severe cases extended to splitting and twisting of the stem (**Figure 4.16**). Double mutant stems appeared succulent and were extremely brittle, due apparently to poor development of vascular tissue. Pedicels of double mutant flowers were also longer than WT, although neither the *fun1* nor the *lv* mutant alone had a visible effect on pedicel length. In addition, in many cases the peduncles of *fun1 lv* plants had small outgrowths

which had the appearance of the early stages of adventitious root growth. Finally, the *fun1 lv* double mutant was extremely slow-growing and showed very weak apical dominance, leading to production of secondary and tertiary lateral branches, and to death of the main shoot apex in a substantial proportion of plants. As a consequence, yield from the double mutant was extremely low, with few plants yielding more than 5 seeds under conditions where WT (and *lv*) plants yielded around 30 and *fun1* plants more than 70 seeds (**Table 4.3**).

4.2.9 Spectral Sensitivity of the *fun1 lv* Double Mutant

The etiolated phenotype of *fun1* mutants under FR has shown that phyA is the only phytochrome with a substantial role in mediation of responses to FR in etiolated pea seedlings. However, continuous R induces substantial de-etiolation in the *lv* mutants, suggesting that at least one phytochrome in addition to phyB is active in mediating the seedling response to R. The results from R pulse experiments (**Figure 4.8**) indicate that phyA can also mediate responses to R, and thus suggest that phyA may also be active under continuous R. The spectral sensitivity of the *fun1 lv* double mutant was therefore examined. **Figures 4.17**A and **4.18** show that the *fun1 lv* double mutant grown in continuous R had much longer internodes, and a greatly reduced leaflet area and rate of node expansion relative to WT plants or either single mutant. The appearance of the *fun1 lv* double mutant under R is very similar to that of a dark-grown WT plant (**Figure 4.18**, also cf **Figures 4.17**A and **4.2**A), with the exception that *fun1 lv* apical buds and leaflets do show some Chl accumulation (**Figure 4.17**A). Whether this represents some degree of induction of Chl synthesis or merely reflects the light-dependent conversion of protochlorophyllide to Chl is not known. In any event, this result clearly demonstrates that both phyA and phyB are active under R, that either phy alone can to a large extent compensate for the absence of the other, and that phyA and phyB together mediate virtually all of the effects of R on stem elongation and leaf development.

However, the fact that the *fun1*, *lv* and *fun1 lv* double mutants grown in WL are all substantially shorter than etiolated WT plants (**Figure 4.15**A) indicates that another photoreceptor in addition to phyA and phyB plays an important role in photomorphogenesis under WL. Furthermore, the near-complete insensitivity of the double mutant to R and FR strongly suggests that B is the active waveband. Both the *fun1* and *lv* single mutants have only a small effect on internode elongation under B (**Figures 4.6** and **3.7**), and **Figures 4.17**B and **4.18** show that the *fun1 lv* mutant also retains a substantial inhibition of stem elongation in response to B. This contrasts with the situation under R (**Figure 4.17**A, **4.18**), and indicates the presence of an additional

photoreceptor with an important role in the mediation of B-induced de-etiolation responses. The effect of *fun1* on leaf development under B is much stronger than that of *lv*, suggesting a more important role for phyA than phyB in control of this response. The fact that the responses of the mutant seedlings to B are closely mirrored in their responses to WL (**Figure 4.15A**) suggests that the B receptor has an important role under high-fluence-rate WL, particularly for the control of stem elongation over the early internodes.

The results in **Figure 4.18** also have some other interesting implications. The de-etiolating effects of R on stem inhibition and leaf development appear to be co-ordinated, insofar as phytochrome deficiency affects both processes to a similar extent. Under B, the de-etiolating effects of phyB are also co-ordinated in a similar fashion, with phyB deficiency causing a small increase in internode length and a small decrease in leaflet area (**Figure 4.18**). In contrast, the loss of phyA strongly decreases leaflet area in seedlings grown under B, but does not cause a corresponding increase in stem elongation. In fact, the loss of phyA causes a small *inhibition* of stem elongation relative to WT (**Figure 4.6, 4.18**) particularly over the early internodes, which is more apparent in the absence of phyB (**Figure 4.18**). This result implies that phyA is actually promoting stem elongation under B, either directly, or through interaction with a B photoreceptor.

4.3 Discussion

4.3.1. Role of PhyA in De-Etiolating Seedlings

Mutant screening under continuous FR has resulted in the isolation of two allelic mutants (*fun1-1* and *fun1-2*) which show a dramatically reduced response to continuous FR (**Figure 4.1B, 4.2**). The *fun1-1* mutant has reduced levels of spectrophotometrically detectable phytochrome (**Table 4.2**), and is strongly deficient in the phyA apoprotein (**Figure 4.7**). These results indicate that the altered photomorphogenesis displayed by the *fun1* mutant lines results from a deficiency in functional phytochrome A. In pea, phyA therefore appears to be the predominant, if not the only, phytochrome mediating FR-induced de-etiolation responses (**Figure 4.6**). This has also been found to be the case for *Arabidopsis* and tomato, in which FR-insensitive, phyA-deficient mutants have also recently been isolated (Parks and Quail 1993, Nagatani et al. 1993; Whitelam et al. 1993; van Tuinen et al. 1995a). In the case of the *Arabidopsis* mutants, phyA deficiency has been shown to result from mutations in the *PHYA* structural gene (Whitelam et al. 1993, Dehesh et al. 1993). The same is also likely to be true for the tomato *fri* mutants, as they map to the same region as the tomato *PHYA* gene (van Tuinen et al. 1996b).

The essentially WT appearance of phyA-deficient pea mutant seedlings grown under continuous WL (**Figure 4.6B**) suggests that, as for *Arabidopsis* and tomato, phyA is largely dispensible for seedling photomorphogenesis under these conditions. However, a slightly elongated phenotype of *fun1-1* seedlings under R (**Figure 4.6**) and under some WL conditions (**Figure 4.9B**) suggested that phyA might play a minor role under R, as also appears to be the case in tomato (van Tuinen et al. 1995a). The subsequent finding that the *fun1 lv* double mutant is substantially more elongated and more etiolated in appearance under R than the *lv* single mutant (**Figure 4.17A, 4.18**) clearly indicates that phyA does in fact have an important role in R induced de-etiolation, but that phyB when present can substitute for phyA in this role to a large extent. A similar conclusion was reached by Reed et al. (1994) in an investigation of phyA- and phyB-deficient mutants of *Arabidopsis*. Although it was initially observed that phyA deficiency in *Arabidopsis* did not affect responses to R (Parks and Quail 1993, Nagatani et al. 1993; Whitelam et al. 1993), the *phyA phyB* double mutant was slightly less responsive to R than the *phyB* single mutant, a difference which was most clearly manifest in the cotyledon expansion response (Reed et al. 1994). Evidence of a role for phyA in control of *Arabidopsis* hypocotyl elongation under R is somewhat less clear (Whitelam et al. 1993, Dehesh et al. 1993, Reed et al. 1994), and the response to very-low-fluence-rate R or to terminal FR pulses (clearly seen in WT and phyB-deficient pea seedlings, **Figure 3.11**) is virtually undetectable in WT *Arabidopsis* (Dehesh et al. 1993; Reed et al. 1994). It therefore appears that phyA may be more important for inhibition of stem elongation by R in pea than in *Arabidopsis*. It also appears that virtually all of the de-etiolation response of pea seedlings to R can be accounted for by the co-action of phytochromes A and B. However, more detailed examination of R responses retained in the double mutant, and molecular characterisation of *fun1* and *lv* mutants will obviously be required to address the possibility that other phytochromes may also have a physiological role in seedlings grown under R.

In pea, both phyA and phyB also appear to have a role in mediation of responses to B (**Figure 4.6**). This might be expected since these phytochromes are known to absorb light in the blue region of the spectrum (Vierstra and Quail 1983, Kunkel et al. 1992). However, removal of both phyA and B does not eliminate the response of pea seedlings to B (**Figure 4.18B**), a result which implies the presence of an additional B-sensing photoreceptor. Physiological evidence for a non-phytochrome photoreceptor mediating the B inhibition of stem elongation in pea seedlings has been described by Laskowski and Briggs (1989), and the presence in pea of a sequence with homology to the *Arabidopsis* cryptochrome gene *CRY1* has recently been reported (Ahmad and Cashmore, 1996). In *Arabidopsis*, phyA, phyB and CRY1 are all involved in the mediation of responses to B (Yanovsky et al. 1995), with phytochrome action predominant under low fluence rates, and cryptochrome under

high fluence rates (Liscum and Hangarter, 1993b). The action of a B photoreceptor is also likely to account for the de-etiolation seen in *fun1 lv* mutant plants under WL.

The increased effectiveness of B for inhibition of stem elongation in *fun1* seedlings is the only result which appears to have no precedent. It is clearly a real effect, since it is seen on both a WT and a phyB-deficient background, and is also seen in mutant seedlings which are strongly deficient in synthesis of the phytochrome chromophore (see **Figure 6.10**). In contrast to pea, phyA-deficient mutants of *Arabidopsis* have slightly longer hypocotyls than WT under B and WL (Whitelam et al. 1993), an effect which, for WL at least, is more pronounced in the absence of phyB (Reed et al. 1994). PhyA-deficient tomato mutants also have longer hypocotyls than WT under B (van Tuinen et al. 1995a). In fact, action of the Arabidopsis B receptor CRY1 appears to be dependent on phytochrome, since B-mediated de-etiolation responses are absent in *phyA phyB* double mutants (Ahmad and Cashmore 1996). These results highlight two differences between pea and *Arabidopsis*; firstly, that phyA in pea mediates a promotory effect of B on stem elongation, and secondly that relatively normal inhibition of elongation by B can be achieved in the absence of both phyA and phyB. These apparent anomalies obviously warrant further investigation, using proven null mutants.

4.3.2. Role of PhyA in Mature Plants

PhyA-deficiency has previously been reported to have little effect on the phenotype of mature, WL-grown *Arabidopsis* and tomato plants. This is true for *fun1* mutant seedlings when grown under continuous light at high R:FR (**Figure 4.6**), but under light with some FR content, mutant seedlings are slightly elongated relative to WT (**Figure 4.9B**). The response of *fun1* to R:FR has yet to be examined, but these observations are consistent with phyA being the phytochrome controlling the inhibition of elongation seen in *lv* mutants grown under low R:FR (**Figure 3.12**). Much more striking is the fact that under our glasshouse conditions, older *fun1* plants show a profoundly pleiotropic phenotype, with reduced stem elongation, increased branching, delayed flowering, retarded fruit development and delayed apical senescence (**Figures 4.3, 4.9, 4.10, Table 4.3**). This phenotype is very similar to that displayed by WT plants grown under non-inductive SD conditions, and represents a developmental orientation away from reproduction in favour of vigourous vegetative growth. It reflects the fact that *fun1* plants are unable to respond to a photoperiod extension (**Figure 4.12-14**), and are thus essentially day-neutral, flowering late in both SD and LD. PhyA deficiency thus results in a dramatic reduction of the photoperiod response for a range of pleiotropic characteristics, a finding which is important because it establishes that phyA is the main if not the only phytochrome

responsible for daylength detection in pea. While phyB does affect flowering in pea there is no clear indication that phyB action is involved with detection of daylength *per se* (see **Section 3.3**). However, the present results indicate that phyA is almost certainly the phytochrome responsible for the HIR-type flower-promotory response previously identified as the main phytochrome response in the control of flowering in pea (Reid and Murfet 1977).

These results are generally consistent with those from *Arabidopsis*, in which phyA-deficiency also results in reduced promotion of flowering in response to a FR-rich photoperiod extension (Johnson et al. 1994) or night-break (Reed et al. 1994), although the virtually normal response of the phyA mutant to day-extension with IL reported by Bagnall et al. (1995) is somewhat at odds with those of Johnson et al. (1994). In addition, flowering in phyA-deficient *Arabidopsis* mutants is promoted to a normal extent by a extension with WFL (Johnson et al. 1994), whereas WFL when supplied as a day extension to the *fun1* mutant had no substantial effect on flowering (**Figure 4.14**). These results therefore suggest that in pea phyA may be the only phytochrome with a substantial role in the promotion of flowering in response to LD, whereas in *Arabidopsis* phyA appears to be less important and at least one other photoreceptor is also active. However, it should be noted that relatively low fluence-rate WFL was used in photoperiod experiments with the *fun1* mutant, and it may be that with WFL fluence-rates comparable to those used in experiments with *Arabidopsis*, flowering in *fun1-1* plants might also be promoted. The slightly greater effectiveness of incandescent compared with WFL in promotion of flowering and associated vegetative changes in the *fun1* mutant does suggest that the mutant does retain some degree of phytochrome action, although whether this is due to residual phyA or to the action of some other phytochrome remains to be determined. At least in the case of stem elongation, the differential effect may be due to the action of phyB, since it is not seen in the *lv-1* mutant (**Figure 4.14**). A more thorough investigation of the effects of *fun1* and *lv* on the control of flowering by photoperiod, EOD-FR and daytime R:FR will obviously be necessary to further clarify the roles of phyA and B. This may be aided by incorporation of these mutants into a later-flowering background such as that provided by a dominant *HR* allele, which confers a near-obligate LD requirement (Murfet 1973). Crosses to obtain *lv HR* and *fun1 HR* genotypes are underway.

Beyond the reports of altered flowering mentioned above, pleiotropic effects of phyA deficiency on the development of mature plants have not been described for either the tomato or *Arabidopsis* mutants. This may be because phyA has a different role in these species, or that the effects of phyA deficiency are less pronounced and have been overlooked. Certainly, the caulescent habit of pea plants is perhaps more conducive to a

consideration of the interrelationship between vegetative and reproductive growth, but it might be expected that the same underlying processes of resource reallocation should be associated with transition to flowering regardless of species. Consistent with this view, photoperiod is known to influence a range of morphological characters in *Arabidopsis* in addition to effects on flowering. For example, Martinez-Zapater et al. (1995) report that the inflorescences of WT *Arabidopsis* plants grown in SD are more highly branched and have shorter internodes than those of plants grown in LD, and that photoperiod also has heterochronic effects on rosette leaf morphology. These are reminiscent of the effects of photoperiod on pea (Murfet 1982), and it would be surprising if these characters did not also differ between WT and phyA-deficient *Arabidopsis* plants grown in extended SD.

4.3.3. PhyA and Graft-Transmissible Effects on Photoperiodism in Pea

The pleiotropic photoperiod response in pea has been extensively studied over many years (e.g. Barber and Paton 1952, Murfet 1971a, 1971b, 1971c) and progress has been thoroughly reviewed on several occasions (Murfet 1977a, 1977b, 1985, 1989). Central to this work has been the characterisation of mutants *sn*, *dne* and *ppd*, which are early-flowering and day-neutral, having lost the capacity for delay of flowering under non-inductive SD conditions (Murfet 1971b, King and Murfet 1985, Arumingtyas and Murfet 1994). The *fun1* mutant is the converse of these mutants in that it is also day-neutral, but is unable to promote flowering under inductive conditions. One of the most interesting aspects of the *sn*, *dne* and *ppd* mutants is that they have readily demonstrable graft-transmissible effects on flowering and other characteristics associated with the photoperiod response (Murfet 1971c, Murfet and Reid 1973, King and Murfet 1985, Taylor and Murfet 1996). Study of these mutants has formed the basis of a model for the photoperiod response in pea which invokes the action of a graft transmissible substance which acts in SD conditions to delay flowering and promote vegetative development (Murfet 1971c, 1977a, 1977b, 1985). Genes *SN*, *DNE* and *PPD* are thought to be genes involved in synthesis of the inhibitor, as mutations at any of the three loci result in a loss of a graft-transmissible flower inhibitory effect. Inhibitor production can be reduced by low temperature treatment (part of the basis of the vernalisation effect, Reid and Murfet 1975) and by exposure to light (Murfet and Reid 1974). It has long been presumed that one or more phytochromes must be responsible for this down-regulation (Reid and Murfet 1977).

The phenotype of *fun1* is consistent with a loss of the light-mediated reduction in inhibitor production, and thus suggests thatphyA is active in the control of inhibitor production. It is therefore expected that graft-transmissible effects of the *fun1* mutant

will be easily demonstrated, as an ability of *fun1* stocks to delay flowering in WT scions under LD, or in *sn, dne* or *ppd* mutant scions under any photoperiod. This expectation is supported by results from preliminary grafting experiments (**Figure 4.19**). The effect of vernalisation on inhibitor production is thought to be more direct than that of light, and has been suggested to result simply from a reduced rate of metabolism in the cotyledons during the cold-temperature treatment. It should therefore be possible to demonstrate clear promotory effects of vernalisation in the *fun1* mutants despite their photoperiod insensitivity. The inhibitor model also predicts that inhibitor synthesis mutants will be essentially epistatic to *fun1*, with the double mutants displaying an early-flowering phenotype in both SD and LD. The production of these double mutants will also help to determine which of the effects of *fun1* in the mature plant are mediated by the inhibitor system. The dwarf phenotype of *fun1* plants is of particular interest, as it points to the intriguing conclusion that phyA can act both to inhibit stem elongation in de-etiolating seedlings, and to promote elongation in older plants. Preliminary results have indicated that the *dne* mutation is indeed epistatic to *fun1* for flowering (**Figure 4.20B**), and relieves many aspects of the *fun1* mature-plant phenotype, including the inhibition of stem elongation (**Figure 4.20C**) and the stem thickening (observations only). These results confirm the involvement of inhibitor in the *fun1* phenotype. Given that *dne* has no effect on seedling de-etiolation under FR (**Figure 3.20A**), these results also indicate the presence of two genetically and physiologically distinct pathways for phyA action, in seedling de-etiolation and in photoperiod detection, respectively. These pathways might be selectively targeted in subsequent mutant screens.

Also of interest are those aspects of the *fun1* phenotype which exceed the phenotype of WT plants grown in SD, such as the stem thickening and pale appearance. While these characteristics could result from a higher inhibitor level in *fun1* relative to SD-grown WT plants, this is difficult to test directly because plants grown in photoperiods shorter than 8 h begin to show signs of photosynthetic limitation. However, the extent of inhibitor involvement with this phenotype could be easily demonstrated using double mutants between *fun1* and *sn, dne* or *ppd*. This is potentially quite an important issue, because if the *fun1* phenotype does result from abnormally high inhibitor level, the anatomical and hormonal changes associated with inhibitor action might be easier to characterise, given their extreme expression in *fun1*.

There is a large body of evidence demonstrating the action of diffusable substances in photoperiodic control of flowering in both LD and SD species, including substances both promotory and inhibitory to flowering. While promotory effects in general appear more prominent in SDP and inhibitory effects in LDP, evidence for both promoters and

inhibitors has been found in both response classes (Zeevaart 1976, Vince-Prue 1985, 1994). In fact much of the evidence can be interpreted to suggest that the photoperiodic control of flowering is determined by a balance of promotory and inhibitory substances (Vince-Prue 1985). Furthermore, a wide variety of grafting experiments have suggested that these floral stimuli may be physiologically similar between related species, and even between more distantly-related species which belong to the same photoperiod response class (Zeevaart 1976, Lang et al. 1977). In pea, in addition to the inhibitor described above, evidence for a graft-transmissible promoter of flowering has also been obtained (Murfet 1971c, 1973), and a gene controlling a graft-transmissible promotory influence has recently been identified (Beveridge and Murfet 1996). Genes with analogous action to the *SN*, *DNE*, and *PPD* group have also been identified in the related LD species *Lathyrus odoratus* (Ross and Murfet 1985a) and *Lens culinaris* (JL Weller and IC Murfet, unpublished data), and trans-specific grafts have suggested that the inhibitory influence is transmissible among these three species (Ross and Murfet 1985b, JL Weller and IC Murfet, unpublished data). These results imply that phyA acts to control flowering in a similar manner in all three species. It would be interesting to examine whether similar effects of phyA are also found in species more distantly related to pea.

Since relatively few phytochrome-deficient mutants have been isolated, and the vast majority of these have been in LDP species (*Arabidopsis*, pea) or in species lacking a clear flowering response (tomato), the involvement of phytochrome in the photocontrol of flowering is better characterised for LDP than for SDP. Many previous physiological reports have focused on the action of a promoter in SDP, and it appears that a LFR-type response may be predominant in governing this promotory influence (Vince-Prue 1985, 1994). However, genes similar to *SN*, *DNE* and *PPD* (i.e. genes required for inhibition of flowering and associated vegetative changes under non-inductive conditions) have also been identified in SDP, such as legumes *Phaseolus vulgaris* (Wallace et al. 1993) and *Glycine max* (Cober et al. 1996). This indicates the presence of an important inhibitory influence in these species and suggests that a inhibitor similar to that in pea is also functioning in SDP, although in these instances the grafting experiments necessary to demonstrate such an activity have yet to be performed. Isolation of phytochrome-deficient mutants and other flowering mutants in an appropriate model SDP species is obviously required as a first step toward an understanding of the genetic differences between the SDP and LDP habits. In light of the fact that at least some of the component responses in photoperiodism are mediated by mobile factors, the development of related, graft-compatible LDP and SDP species as model systems might be particularly useful.

4.4. Methods

4.4.1. Mutagenesis and Mutant Screening

Approximately 1500 seeds of WT cv. Torsdag were imbibed in 1L of a 1% (v/v) solution of ethylmethanesulphonate for 6 h at room temperature (approx. 18°C, 1000 seed) or at 25°C (500 seed). These treatments were at the severe end of the range employed in previous EMS mutagenesis of pea (Kneen and LaRue 1988, Duc and Messager 1989), and were chosen in an attempt to maximise the mutation rate, in view of the limited space available for growth of M_1 plants. After rinsing for a further hour in running water, seeds were planted in standard pea potting mix. These plants (M_1 generation) were grown to maturity under an 18-h photoperiod in the glasshouse. M_2 seed from individual plants was harvested and retained for screening. Some 12% of plants from the 18°C treatment and 60% of the plants from the 25°C treatment failed to produce a single seed. M_2 seed was planted in standard pea potting mix in plastic toteboxes and grown for 7 days under 8 μmol m^{-2} sec^{-1} FR, in batches of about 700. Each family was represented by no more than 8 individuals. After 7 days, toteboxes were transferred to the glasshouse, where selected mutants were allowed to de-etiolate for several days before transplanting to pots.

4.4.2. Other Methods

For experiments in **Figures 4.7, 4.8** and **Table 4.2**, plants were grown at 25°C in vermiculite. All other plants were grown in standard Hobart pea potting mix, either at 20°C in growth cabinets (**Figures 4.1, 4.2, 4.6, 4.15, 4.17, 4.18, 4.20A**) or in a heated glasshouse (**Figures 4.3-4.5, 4.9-4.11, 4.16, 4.20**) or phytotron (**Figures 4.12-4.14, 4.19**). The photoperiod experiment (**Figure 4.13**) was performed as described in section **3.4.1**, except that extensions were given with 10 μmol m^{-2} sec^{-1} light from incandescent globes or from cool-white fluorescent tubes. Standard Hobart light sources were used for all experiments, with the exception of the R pulse experiment (**Figure 4.8**) in which standard RIKEN light sources were used. Methods used for immunodetection of phytochrome (**Figure 4.7**) and for *in vivo* spectrophotometry (**Table 4.2**) were identical to those described in **Chapter 2**. Graft scions (**Figure 4.19**) were prepared by decapitating six-day-old seedlings at the epicotyl 5-10 mm above the cotyledonary node, and trimming the cut end to form a wedge. Stocks were prepared by decapitating eleven-day-old seedlings between the fourth and fifth node and making a longitudinal slit bisecting the stem. The scion wedge was introduced into this slit and secured with a small rubber band. After grafting, plants were covered with plastic bags for one week to minimise dehydration prior to establishment of the graft union.

Table 4.1. F_2 segregation data for *fun1* and linkage group I/II markers

Loci[a]		Phenotype[b]				n	Chi-squared Locus 1	Chi-squared Locus 2	Chi-squared Joint	Linkage Prob.	Recomb. Fraction	SE
		DD	DR	RD	RR							
fun1	*a*	215	52	53	44	364	0.53	0.37	24.56	< 0.0001	33.6	9.2
fun1	*wb*	199	68	84	13	364		1.47	17.44	<0.0001	38.9	4.1
fun1	*k*	190	77	86	6	359		0.68	18.96	<0.0001	26.8	5.1
wb	*k*	258	21	18	62	359			204.95	<0.0001	12.0	2.6
a	*k*	202	64	74	19	359			0.01	>0.9	----	----
a	*wb*	208	60	75	21	364			0.51	>0.4	----	----

[a] Cross AF140 (*fun1-1 a WB K*) x L111 (*FUN1 A wb k*).

[b] D = dominant, R = recessive. The first -named locus is listed first.

Table 4.2. *In vivo* spectrophotometric measurement of phytochrome in WT cv. Torsdag and phytochrome-deficient mutant seedlings.

	$\Delta\Delta A_{(730\text{-}800nm)}$	
Genotype	Dark	4 h R
WT	20.7±1.2	3.0±0.3 (14.5%)
fin1-1	0.6±0.2	0.5±0.1 (2.4%)
pcd2	0.2±0.1	0.2±0.1 (<1.0%)
lv-5	18.9±2.1	2.8±0.1 (13.5%)

Phytochrome content was measured in etiolated seedlings after 5 days growth in complete darkness, or after an additional 4-h exposure to R (17 μmol m^{-2} sec^{-1}). n=4. Figures in parentheses indicate phytochrome content relative to etiolated WT seedlings.

Table 4.3. Phenotype of *fun1-1* plants grown in LD

Character	Genotype	
	WT	*fun1-1*
Length between nodes 1 and 12 (cm)	80.4±0.8	56.9±1.0
Node of flower intiation (NFI)	16.5±0.1	19.0±0.3
Node of flower development (NFD)	16.5±0.1	24.1±0.8
Flower/leaf relativity index	-0.65±0.02	-1.92±0.09
Peduncle length at NFD (cm)	10.4±0.2	18.7±0.8
Total nodes at apical arrest (TN)	22.1±0.2	41.2±0.6
Reproductive nodes (TN-NFI+1)	5.7±0.2	23.2±0.7
Seed yield	29.6±0.6	71.1±2.4

Characters were measured in WT (n=76) and *fun1-1* (n=28) segregates in the F_2 of the cross TOR x AF140. All plants were grown under an 18-h photoperiod under standard glasshouse conditions.

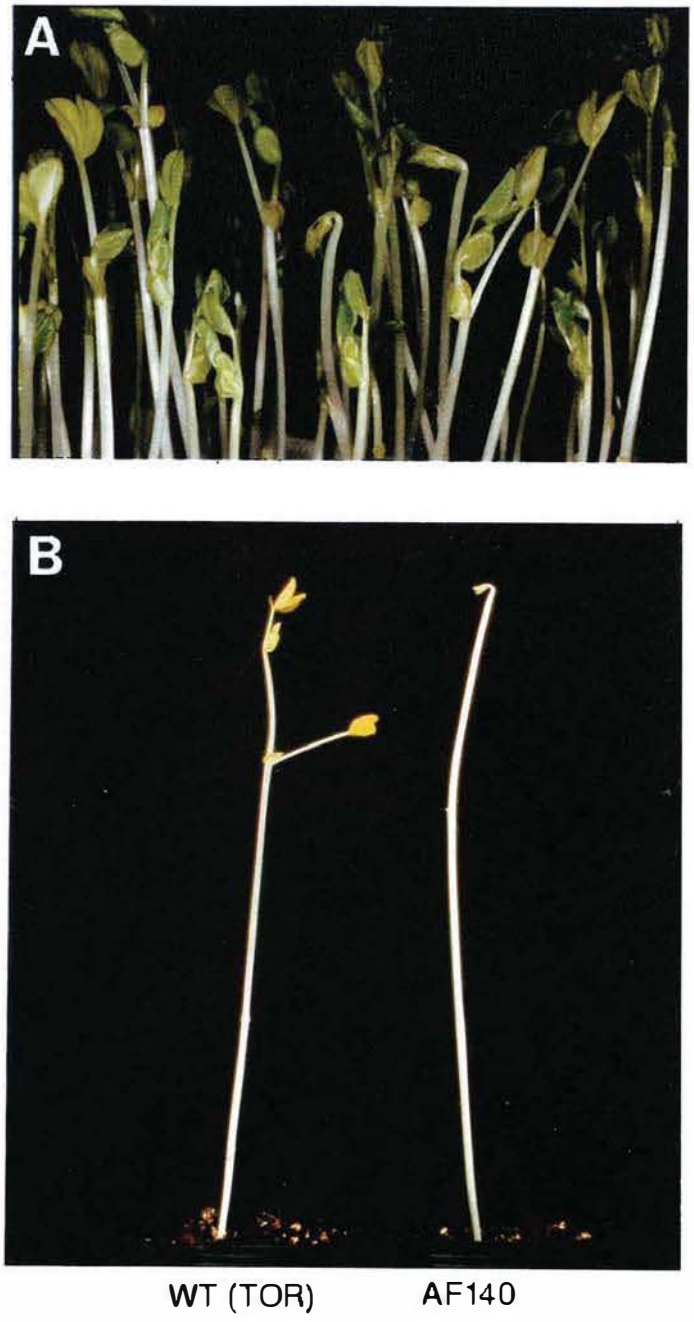

Figure 4.1. Selection of mutants with reduced response to FR. **A.** Mutant screen showing mutants retaining an apical hook under FR (8 μmol m^{-2} sec^{-1}). **B.** Phenotype of FR-insensitive mutant line AF140 grown under FR (12 μmol m^{-2} sec^{-1}) for 7 days at 25°C.

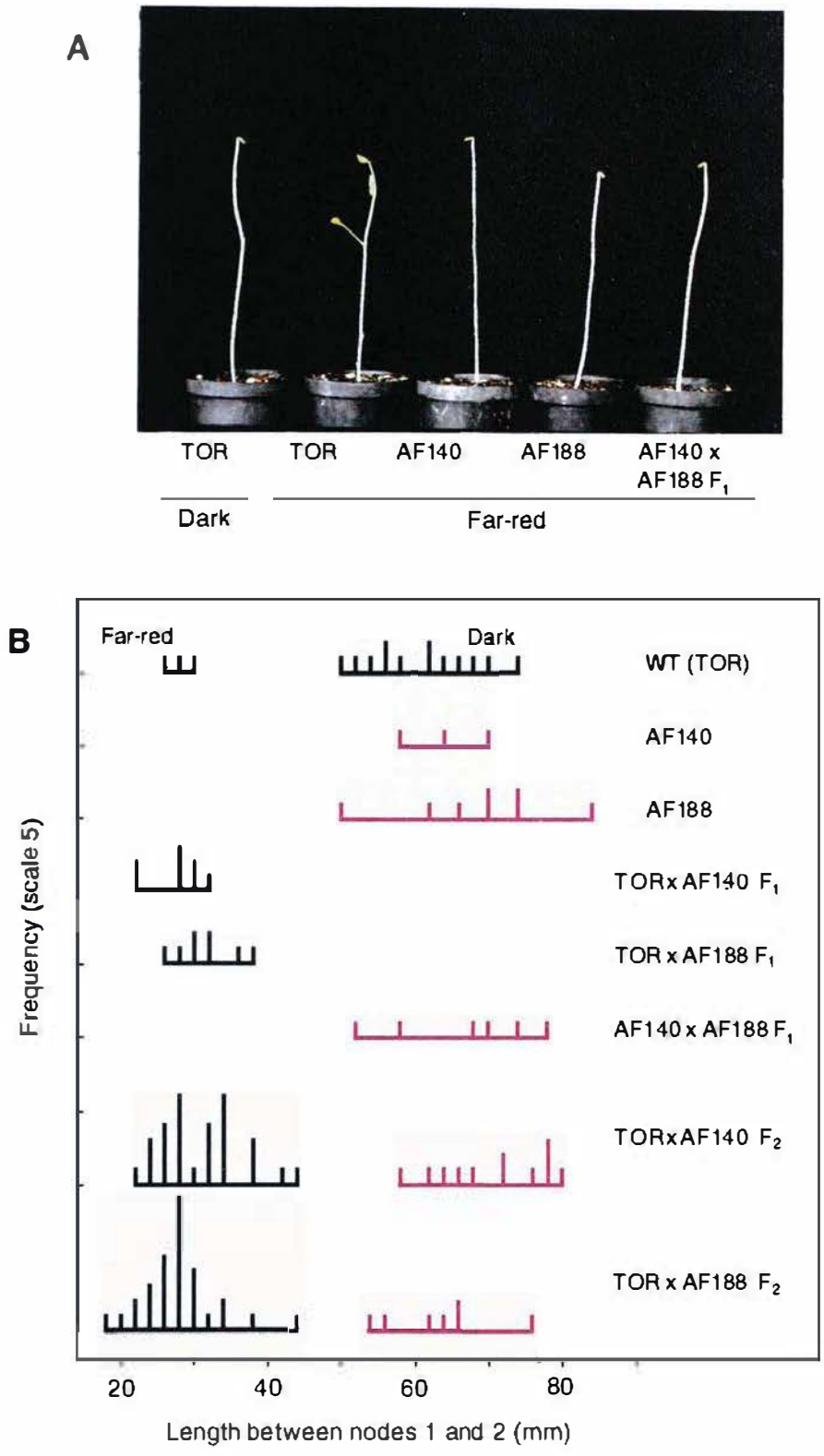

Figure 4.2. Genetic analysis of FR-insensitive mutants AF140 and AF188. A. Photograph of representative plants. B. Frequency distributions for internode length. Black and purple bars indicate plants with WT and *fun1* phenotype, respectively.

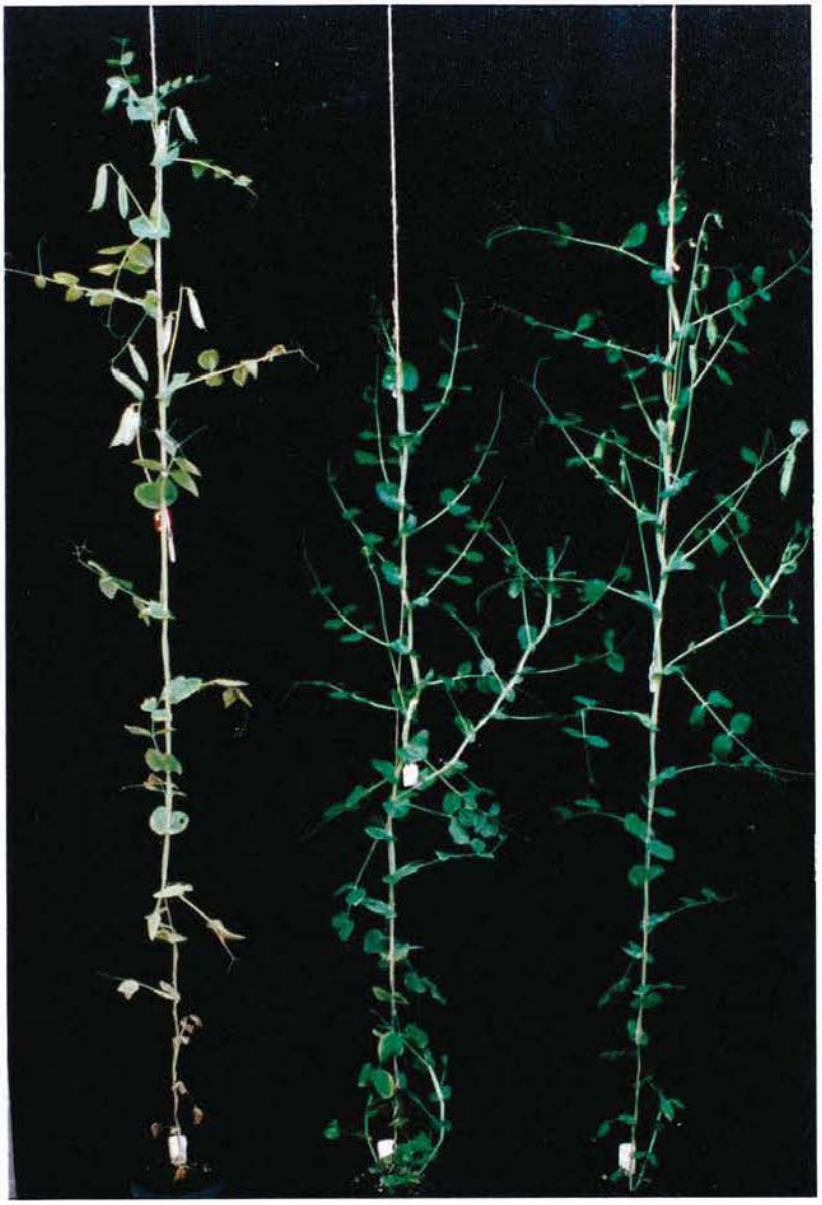

Figure 4.3. Phenotypes of mature *fun1* mutant plants. WT cv. Torsdag, *fun1-1* and *fun1-2* plants grown for eight weeks in long days (18 h) under standard glasshouse conditions.

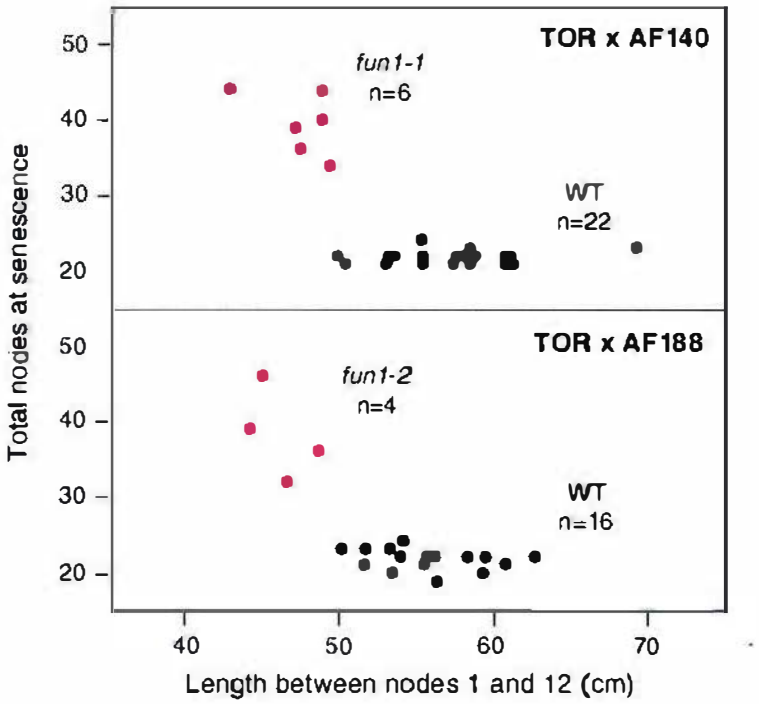

Figure4.4. Co-segregation of the *fun1* late-senescing phenotype with reduced internode length. Lines AF140 (*fun1-1*) and AF188 (*fun1-2*) were crossed to WT cv. Torsdag (TOR) and resultant F_2 populations were grown in long days (18 h) under standard glasshouse conditions. Black and purple points represent plants with WT and *fun1* phenotypes, respectively.

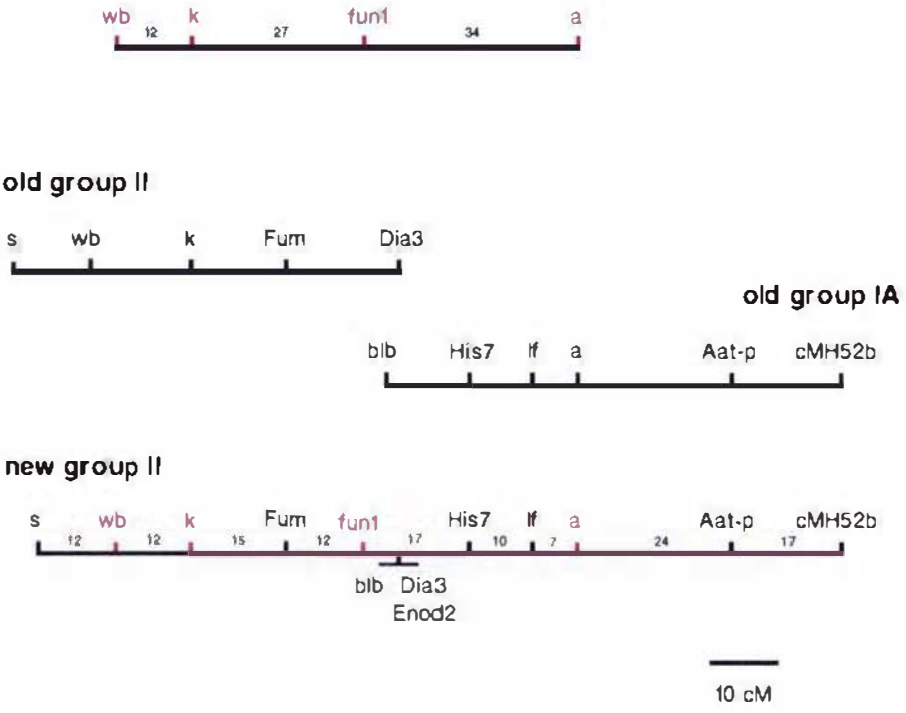

Figure 4.5. Map location of the *fun1* gene in linkage group II. At top is the map generated by the data in Table 4.1. This is aligned with recent maps of group II and group IA (Weeden et al. 1993). A new composite map for group II is shown below.

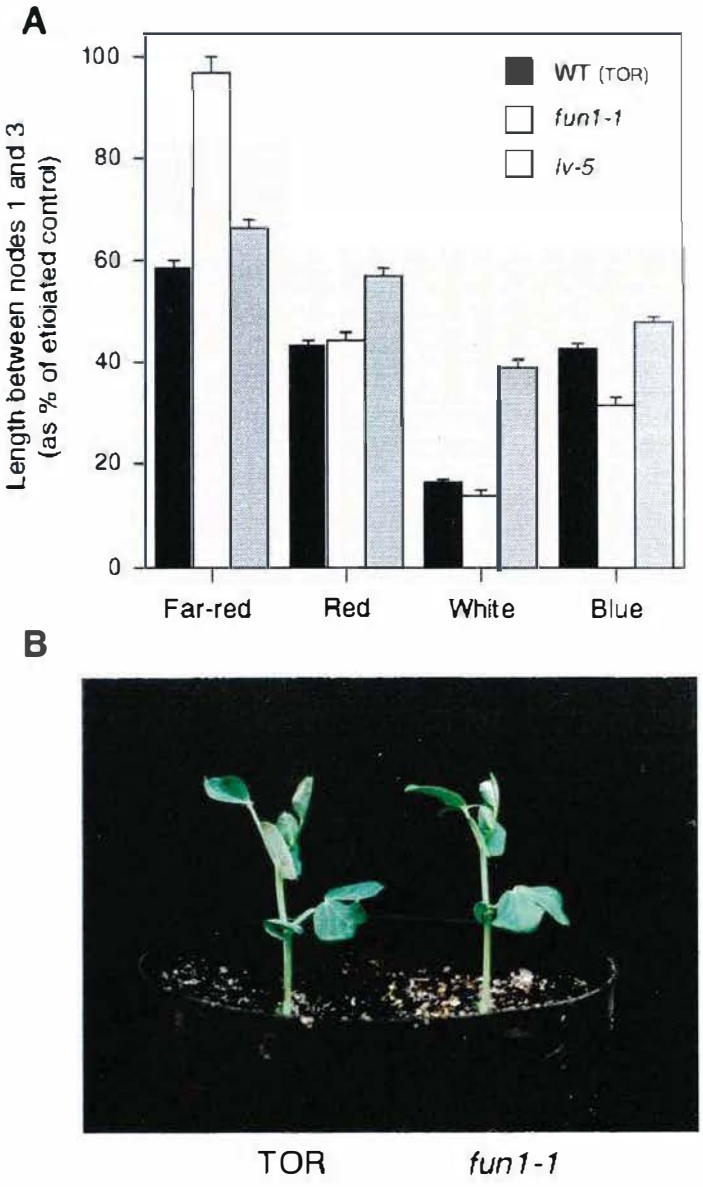

Figure 4.6. A. Stem elongation of the *fun1-1* mutant grown under continuous monochromatic light. The *lv*-5 mutant is included for comparison. Growth conditions; FR (8 μmol m^{-2} sec^{-1}), WFL (140 μmol m^{-2} sec^{-1}) R (20 μmol m^{-2} sec^{-1}) B (10 μmol m^{-2} sec^{-1}), 20°C. Bars indicate SE, n = 10 to 12. **B.** Photograph of 10-day-old WT and *fun1-1* plants grown under continuous WFL.

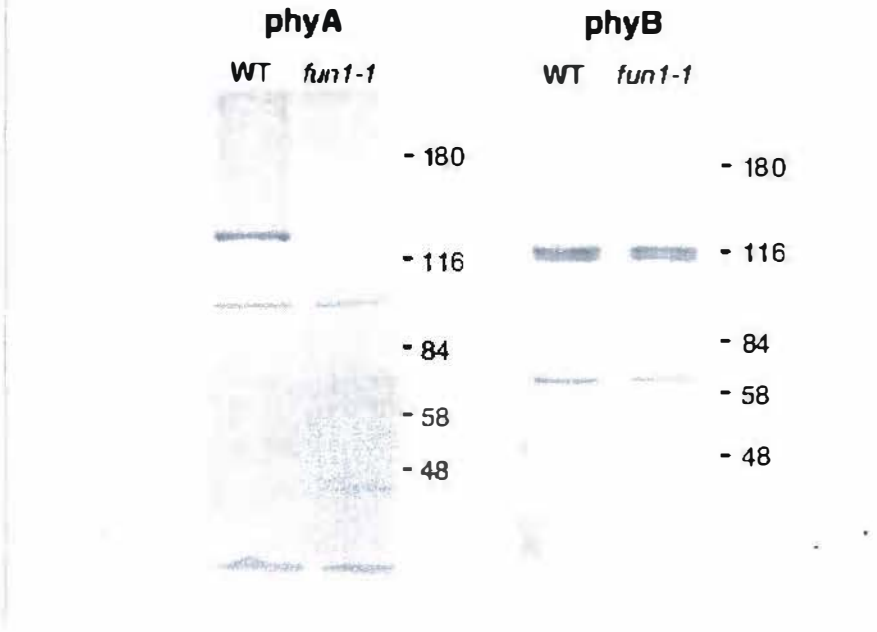

Figure 4.7. Immunoblot detection of PHYA and PHYB apoprotein in crude extracts of etiolated WT and *fun1-1* mutant plants. Each lane contains extract equivalent to 2.5 mg fresh weight. The positions and molecular masses (kD) of prestained markers (Sigma) are indicated. PHYA and PHYB were detected using monoclonal antibodies mAP5 (Nagatani et al. 1984) and mAT5 (Lopez-Juez et al. 1992), respectively.

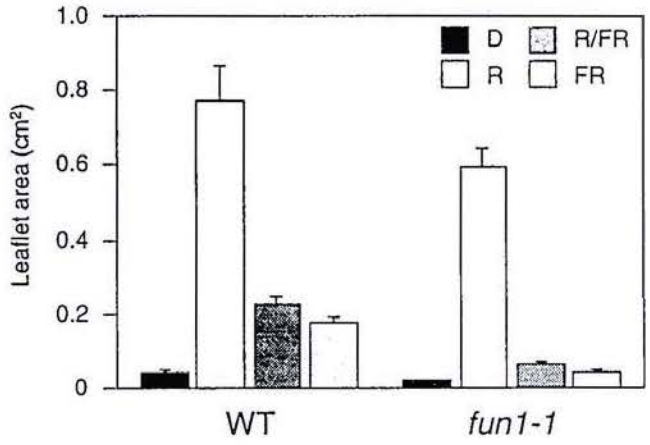

Figure 4.8. R/FR reversibility of de-etiolation in WT and *fun1* seedlings. WT cv. Torsdag and *fun1-1* mutant plants were given saturating pulses of R (17 μmol m^{-2} sec^{-1}, 10 min), FR (12 μmol m^{-2} sec^{-1}, 15 min), or R followed by FR (R/FR) at 4-h intervals for 10 days after sowing, or maintained in complete darkness (D).

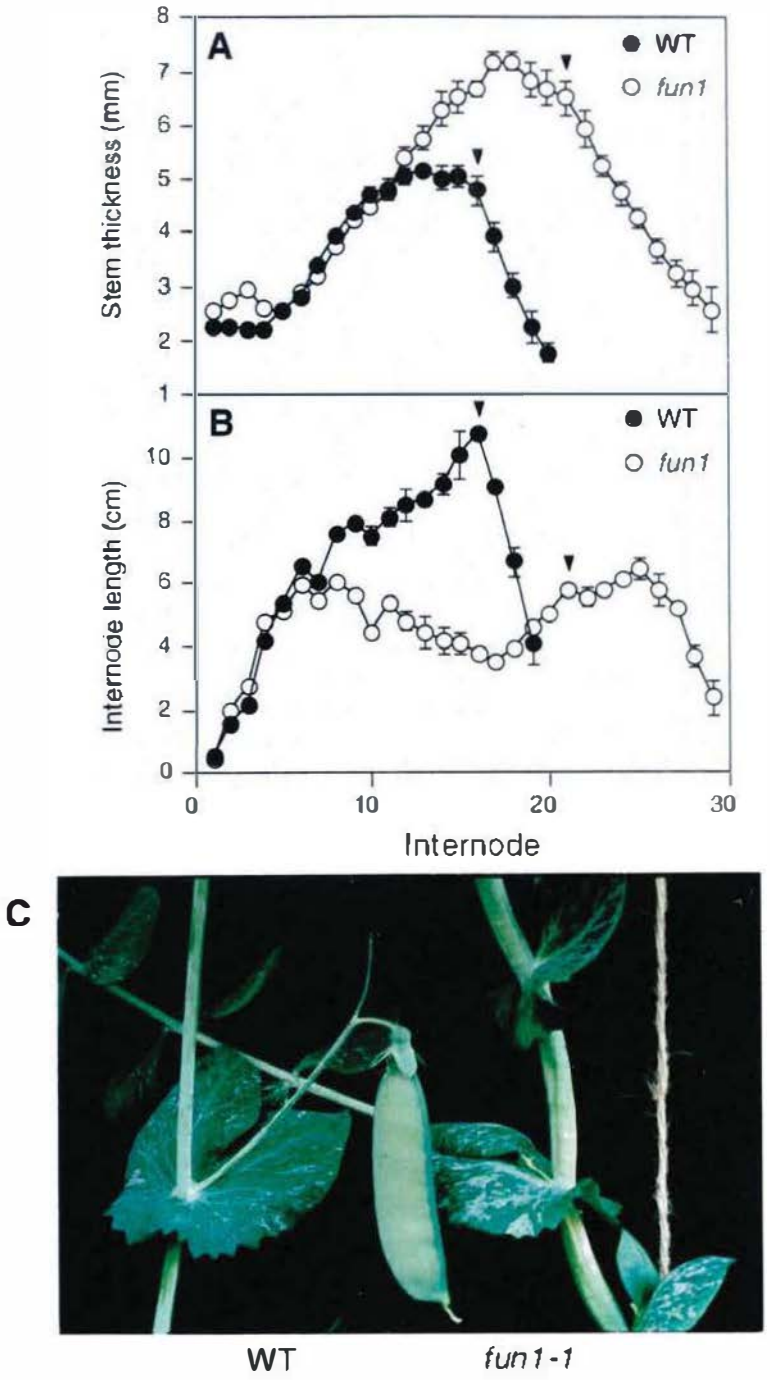

Figure 4.9. Effects of *fun1-1* on stem thickness and internode length. A. Mean stem thickness. **B.** Mean internode length. Bars indicate SE, n = 6. Arrows indicate node of flower initiation. C. Photograph showing the stem of a wild-type and a *fun1-1* plant at the node of flower initiation, showing the thickened stem with transverse banding, and an aborted flower intial in *fun1-1*. Growing conditions; 18-h photoperiod under standard glasshouse conditions.

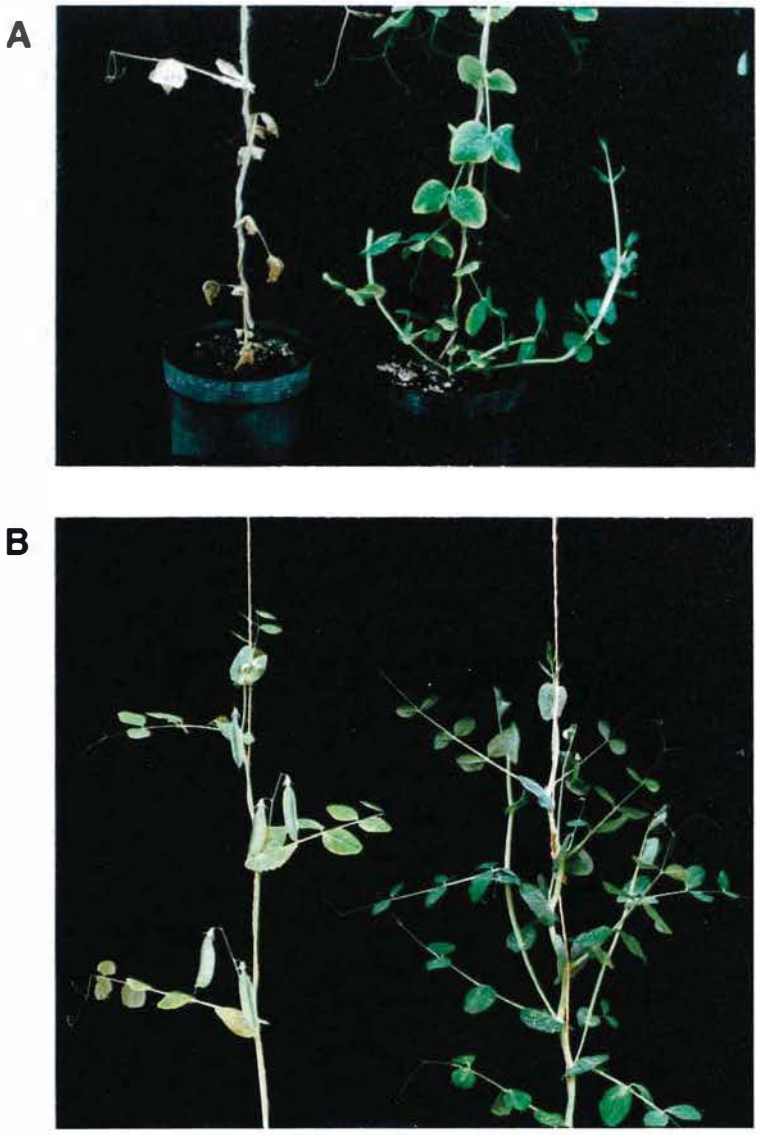

Figure 4.10. Increased lateral branching in the *fun1-1* mutant. Representative WT cv. Torsdag (left) and *fun1-1* plants are shown. **A.** Basal branching. Lateral branches have been decapitated. **B.** Aerial branching. Growing conditions; 18-h photoperiod under standard glasshouse conditions.

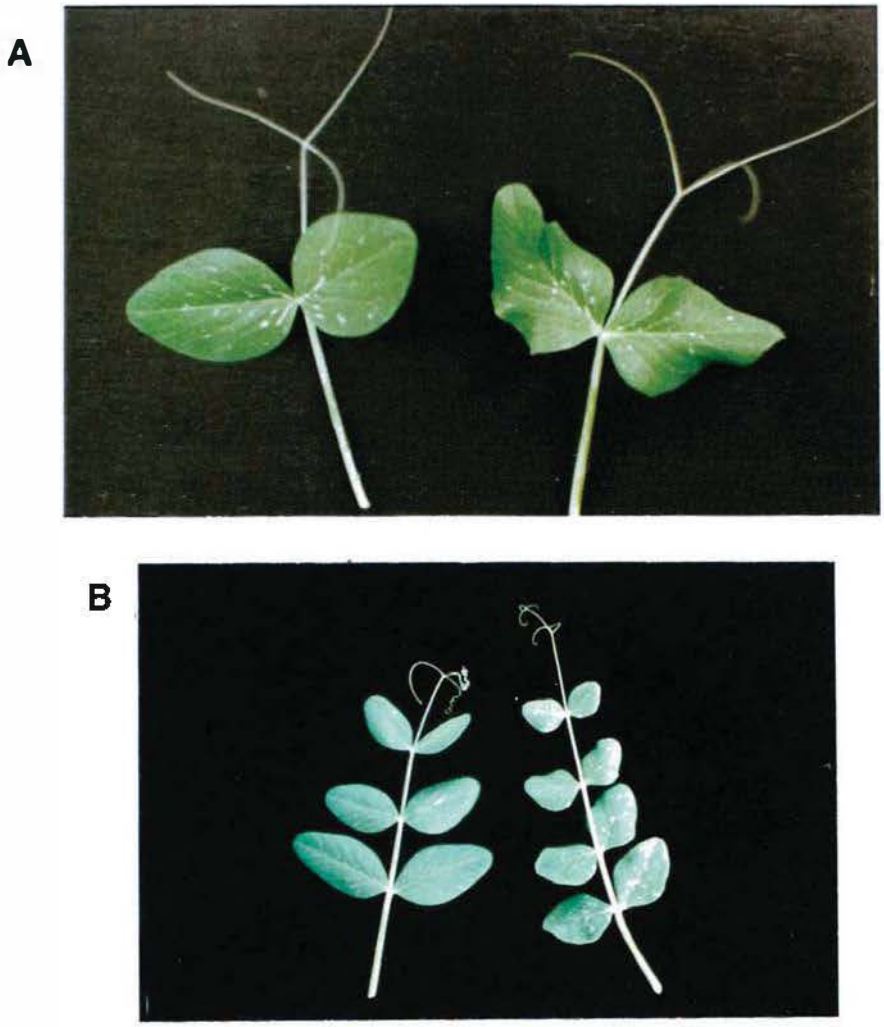

Figure 4.11. Leaf phenotypes of *fun1* plants. **A.** Leaf at node 6 from representative WT cv. Torsdag (left) and *fun1-1* plants. **B.** Comparison of maximum leaf complexity seen in WT (left) and *fun1-1* plants. Growing conditions; 18-h photoperiod under standard glasshouse conditions.

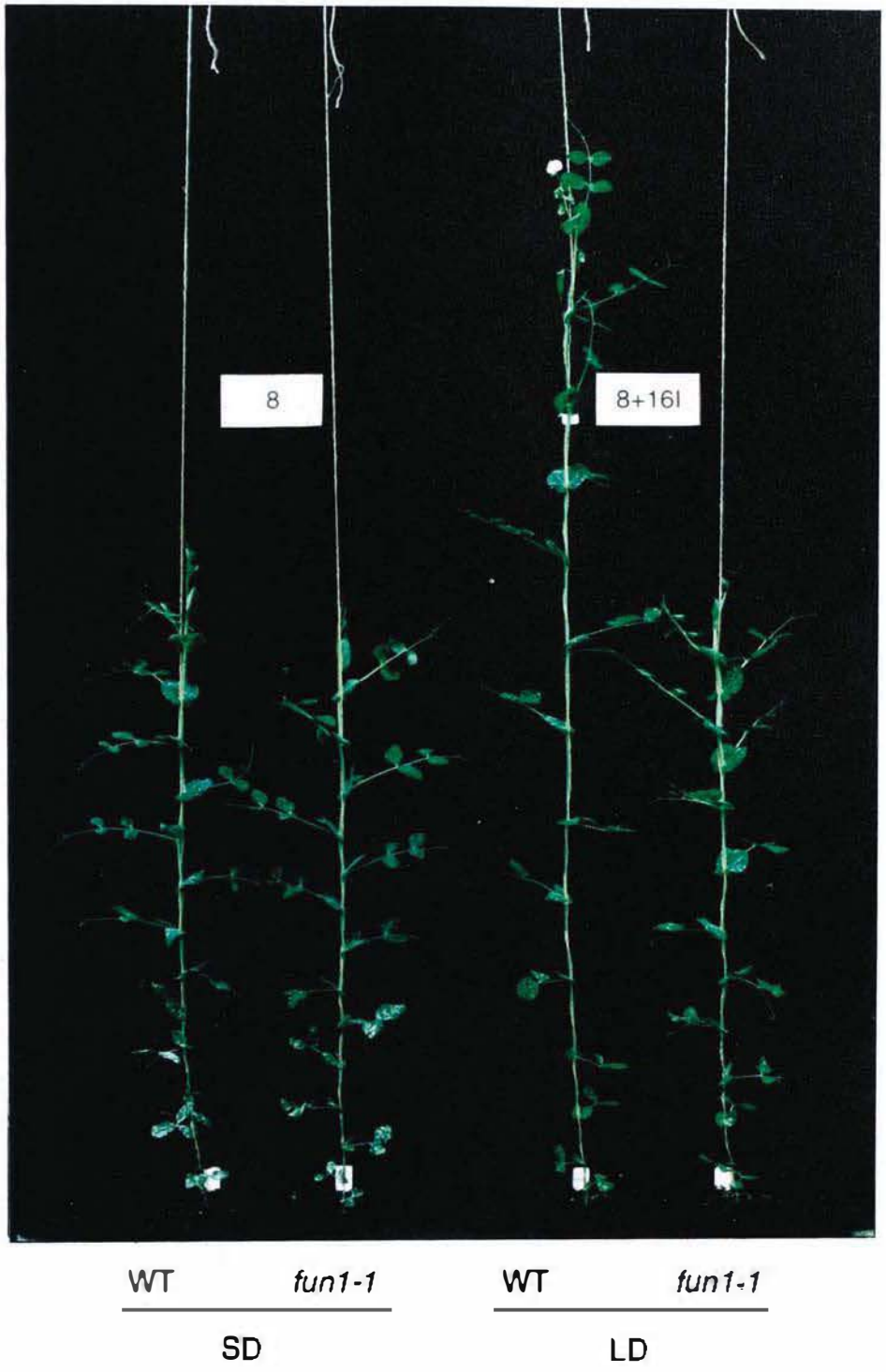

Figure 4.12. Response of the *fun1* mutant to a photoperiod extension. WT cv. Torsdag and *fun1-1* plants were grown for 6 weeks from sowing in an 8-h photoperiod of natural daylight with or without a 16-h extension given as weak incandescent light (10 μmol m^{-2} sec^{-1}).

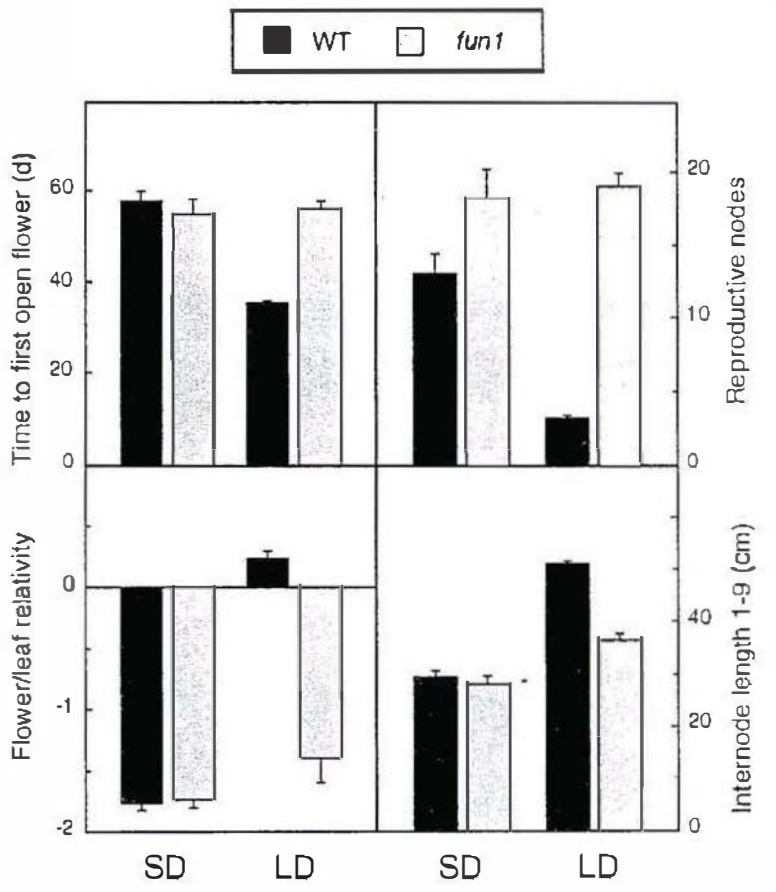

Figure 4.13. Responses of the *fun1* mutant to a photoperiod extension. Plants were grown from sowing in an 8-h photoperiod of natural daylight with (LD) or without (SD) a 16-h extension given as weak incandescent light (10 μmol $m^{-2}s^{-1}$). Bars indicate SE, n = 6-8.

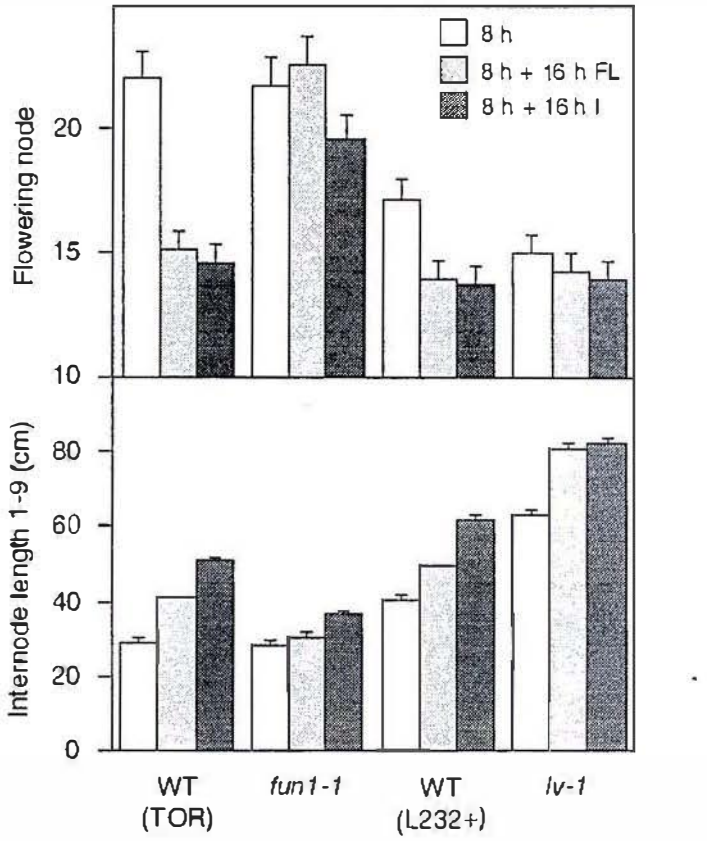

Figure 4.14. Responses of the *fun1-1* and *lv-1* mutants to photoperiod extensions with light of high or low R:FR. Plants were grown from sowing in an 8-h photoperiod of natural daylight with or without a 16-h extension, of either incandescent (I) light (10 μmol $m^{-2}s^{-1}$; R:FR≈0.6) or fluorescent (FL) light (10 μmol $m^{-2}s^{-1}$; R:FR=4.8). Bars indicate SE, n = 6-8.

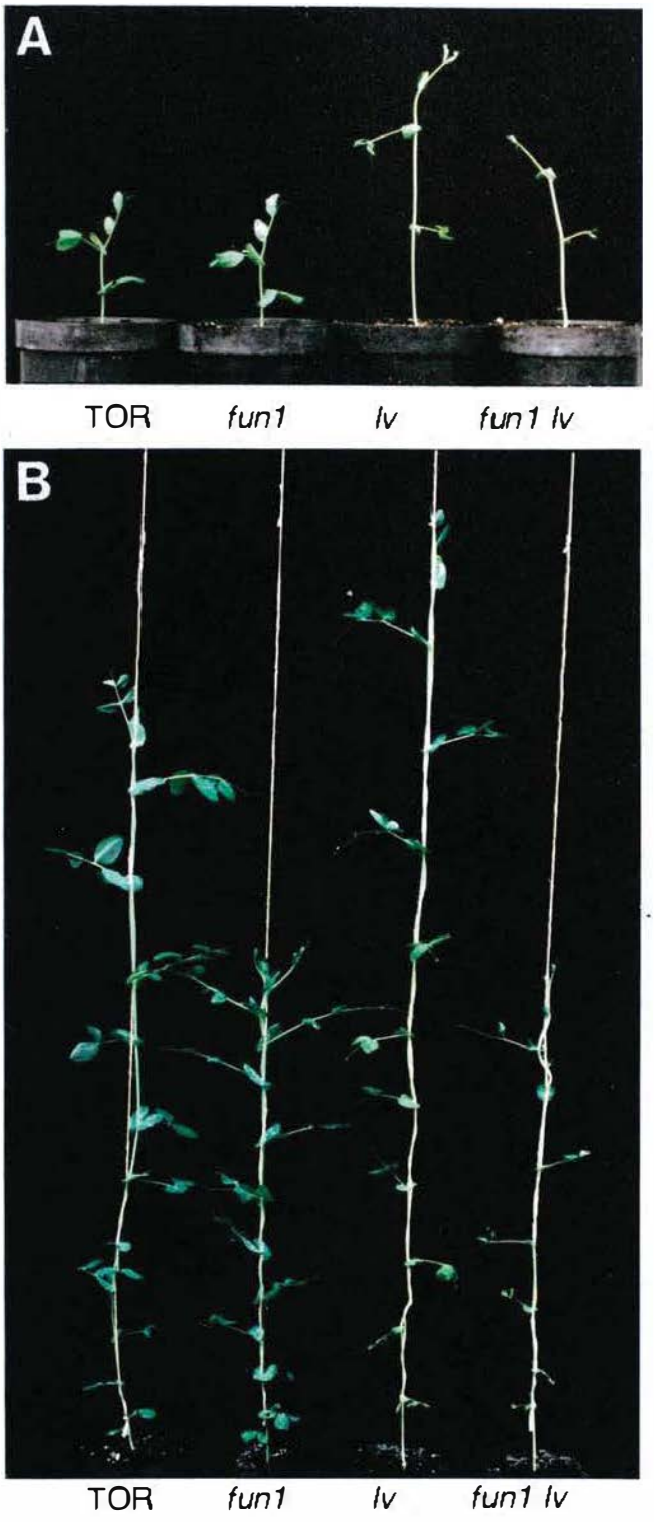

Figure 4.15. Phenotype of *fun1*, *lv* and *fun1 lv* double mutants. **A.** 11-day-old WT cv Torsdag (TOR) and mutant seedlings grown under WFL (150 μmol m^{-2} sec^{-1}). **B.** 7-week-old plants grown in standard glasshouse conditions (18-h photoperiod). Mutant plants shown are representative segregates from the F_3 of the cross AF140 (*fun1-1*) x AF280 (*lv-5*).

Figure 4.16. Phenotype of the *fun1 lv* double mutant. The photograph shows the upper part of the shoots of representative *fun1-1* and *fun1-1 lv-5* plants grown for seven weeks in an 18-h photoperiod under standard glasshouse conditions.

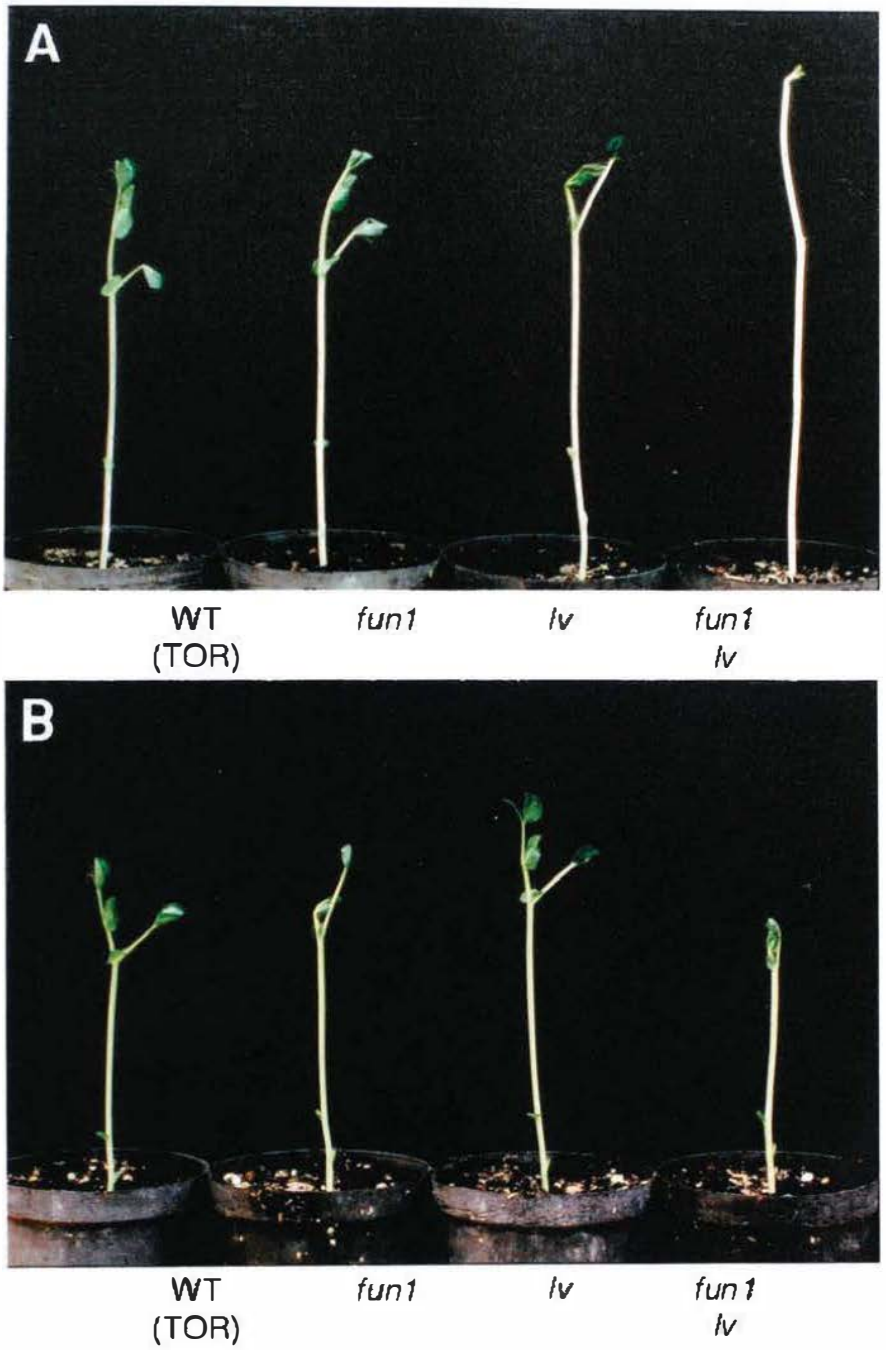

Figure 4.17. Phenotypes of *fun1*, ***lv*** and *fun1lv* double mutants under monochromatic light. **A.** Seedlings grown under R (20 μmol $m^{-2}s^{-1}$). **B.** Seedlings grown under **B** (10 μmol $m^{-2}s^{-1}$). Photographs taken 8 days after sowing.

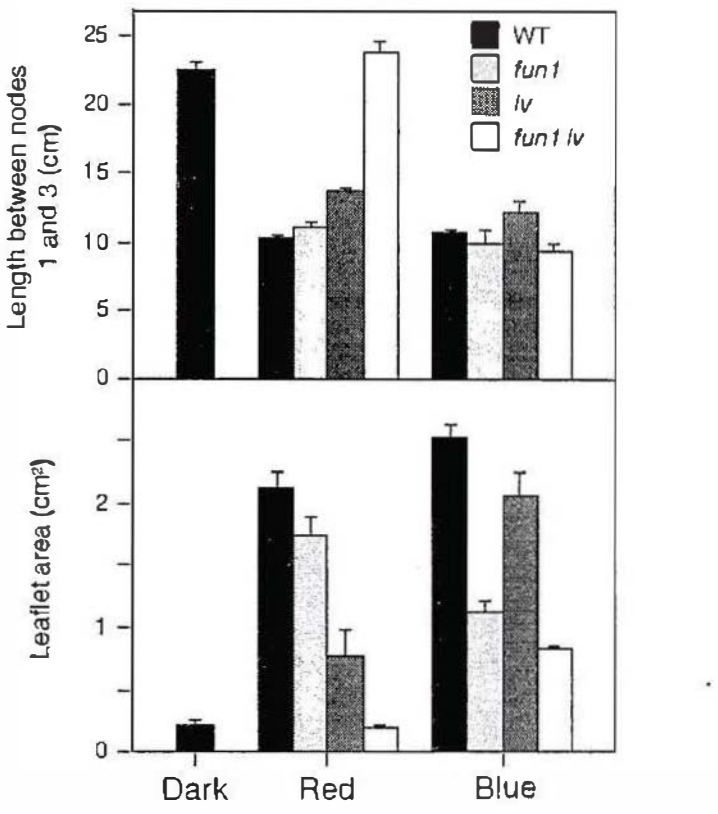

Figure 4.18. Phenotype of *fun1*, *lv* and *fun1 lv* double mutants under monochromatic R (20 μmol $m^{-2}s^{-1}$) or B (10 μmol $m^{-2}s^{-1}$). Leaflet area was estimated as the product of the length and width of a leaflet from the first true foliage leaf (node 4). Bars indicate SE, n = 6-8.

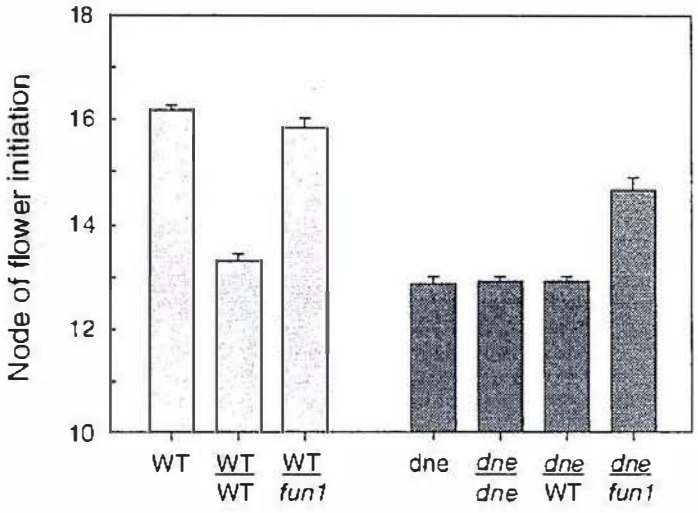

Figure 4.19. Graft-transmissible inhibitory effects of the *fun1* mutant on flowering in WT and *dne* mutant scions held in a 24-h photoperiod. Epicotyls of six-day-old seedlings were wedge-grafted into the fourth internode of eleven-day-old seedlings in the genotype combinations indicated. Bars represent SE, n=8-12. Growing conditions; 8-h natural photoperiod extended with 16 h weak incandescent light (3 μmol m^{-2} sec^{-1}).

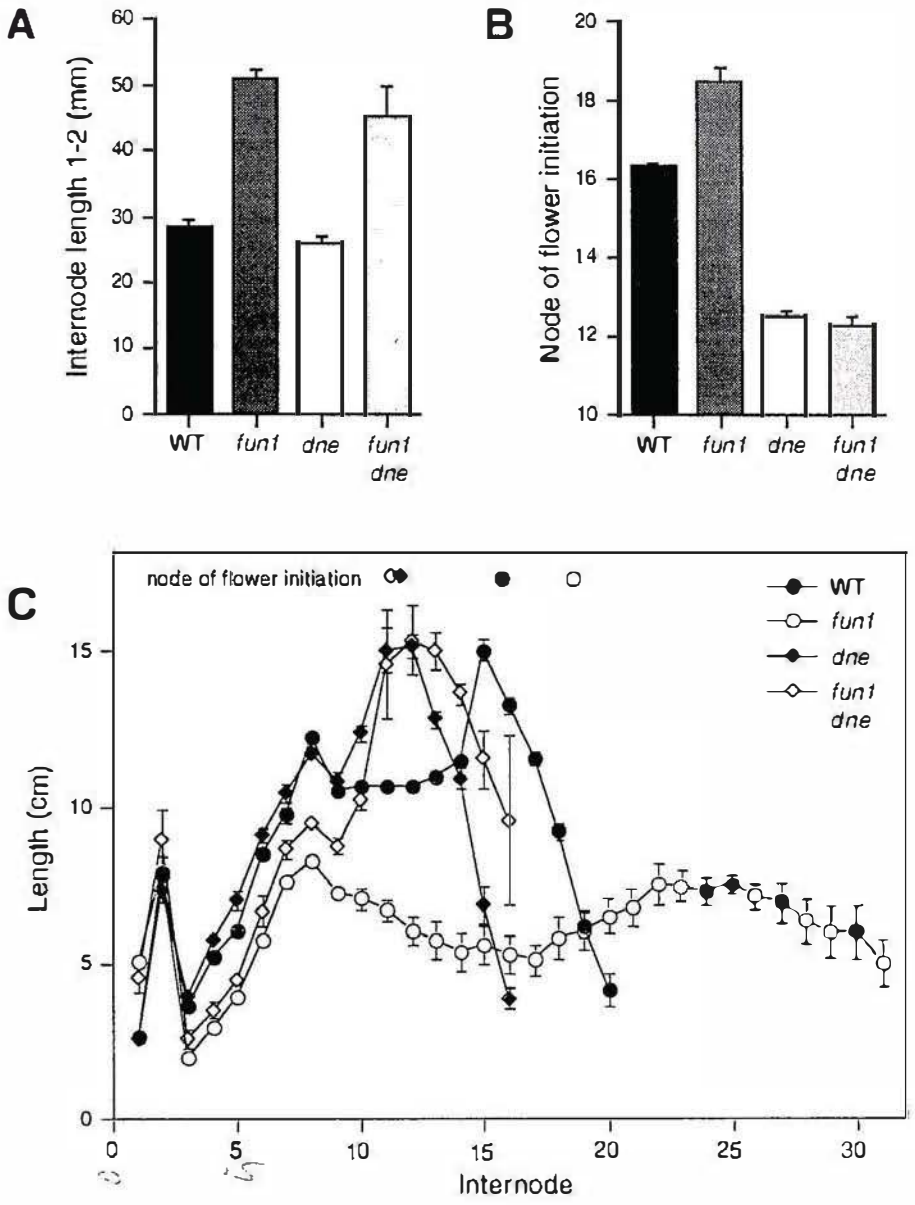

Figure 4.20. Interaction of *fun1* with the early-flowering, day-neutral *dne* mutant. An F_2 population of the cross AF140 (*fun1-1*) x K218 (*dne*) was grown under FR for 7 days and then transferred to an 18-h photoperiod until senescence. *fun1 dne* double mutants were identified as a distinct class of FR-unresponsive, early-flowering segregate. Values given are mean values for all segregates in each class, with n=57, 18, 17 and 4 for WT, *fun1*, *dne* and *fun1 dne*, respectively. **A.** Length between nodes 1 and 2 for plants grown under FR. **B.** Node of flower initiation. **C.** Internode lengths. The mean node of flower initiation for each class is indicated by the plot symbols at top. Growing conditions; FR, 8 μmol m^{-2} sec^{-1}, 20°C. Plants were grown to maturity under standard glasshouse conditions.

5. Mutants Deficient in the Phytochrome Chromophore: The *pcd1* Locus

5.1. Introduction

As described in Chapter 1, the spectral characteristics of phytochrome derive from an interaction between an apoprotein and a covalently bound linear tetrapyrrole chromophore (Lagarias and Rapoport 1980). Holophytochrome biosynthesis therefore requires the convergence of two separate pathways, one for synthesis of the apoprotein and another for synthesis of the chromophore precursor, phytochromobilin (PΦB). Assembly of apophytochrome with PΦB is an autocatalytic process that is thought to occur in the cytoplasm (Lagarias and Lagarias 1989, Terry and Lagarias 1991). There is no evidence for the involvement of any other protein in phytochrome assembly. Since assembly to phycocyanobilin (PCB), a structural analog of PΦB, proceeds at the same rate for both PHYA (Li and Lagarias 1992) and PHYB apoproteins (Kunkel et al. 1993), it is likely that at least these phytochromes (and possibly PHYC as well; Quail et al. 1995) use the same chromophore.

Figure 1.5 shows the proposed pathway for PΦB synthesis in higher plants, which has been developed on the basis of metabolism studies (Elich and Lagarias 1987, Elich et al. 1989, Terry et al. 1995) and by analogy with the pathway for the synthesis of related bilins in algae (Beale 1993, Terry et al. 1993b). Synthesis of PΦB appears to occur entirely in the plastid (Terry and Lagarias 1991, Terry et al. 1993b) where it is derived from 5-aminolevulinic acid and is synthesised via a pathway which branches from the synthetic pathway for chlorophyll. The first committed step for PΦB synthesis is thought to be the oxygenation of heme to biliverdin (BV) IXα, which is subsequently reduced to 3*Z*-PΦB. 3*Z*-PΦB is further isomerised to 3*E*-PΦB which is considered to be the natural chromophore precursor (Cornejo et al. 1992, Terry et al. 1995). Although much progress has been made recently, understanding of this pathway in higher plants is still far from complete. For example, although ferrochelatase has been cloned (Smith et al. 1994) and PΦB synthase activity has been characterised (Terry and Lagarias 1991, Terry et al. 1995), direct evidence for heme oxygenase and PΦB isomerase activities has yet to be demonstrated (Terry et al. 1993b, 1995).

Since the advent of a mutant-based approach to plant photomorphogenesis, many mutants that alter phytochrome function have been identified and characterised (Koornneef and Kendrick 1994). These include mutants deficient in specific phytochromes. Study of these mutants has established that phyA, the light-labile phytochrome abundant in etiolated seedlings, is specifically involved in the mediation of seedling responses to far-red light (FR) (**Chapter 4**; also Nagatani et al. 1993, Parks and Quail 1993, Whitelam et al. 1993, van Tuinen et al. 1995a), while the light-stable B-type phytochromes appear to control responses to red light (R) (**Chapter 3**; also Somers et al. 1991, Devlin et al. 1992, López-Juez et al. 1992, van Tuinen et al. 1995b).

A third class of mutant, deficient in response to both R and FR, is represented by the long hypocotyl *hy1*, *hy2*, and *hy6* mutants of *Arabidopsis* (Koornneef et al. 1980, Chory et al. 1989a). In view of what is known from phyA- and phyB-specific mutants, the co-incidence of R and FR insensitivity in *hy1*, *hy2*, and *hy6* suggests that the lesions in these mutants affect the activity of both phyA and phyB. Etiolated seedlings of *hy1*, *hy2*, and *hy6* have strongly reduced levels of spectrally active phytochrome but WTor near- levels of PHYA apoprotein (Koornneef et al. 1980, Chory et al. 1989a, Parks et al. 1989), suggesting that phytochrome apoprotein is synthesised normally in these mutants, but for some reason is not converted into a spectrally active holoprotein. The most plausible explanation, that the mutants are deficient in the phytochrome chromophore, is further supported by the demonstration that exogenously supplied BV can fully or partially restore a WTphenotype in the *hy1*, *hy2*, and *hy6* mutants (Parks and Quail 1991, Nagatani et al. 1993). Similar mutants are also known in tomato. Like *hy1*, the *aurea* (*au*) and *yellow-green-2* (*yg-2*) mutants are insensitive to both R and FR and have strongly reduced levels of spectrally active phytochrome (Koornneef et al. 1985, Parks et al. 1987, Kendrick et al. 1994). Attempts to rescue the *au* mutant by feeding chromophore precursors or structural analogues has so farproven unsuccessful (Kendrick et al. 1994). However, the phenotypic similarity of *au* to the chromophore-deficient *Arabidopsis* mutants and epistasis of *au* and *yg-2* over a constitutively expressed *PHYA* transgene are consistent with the hypothesis that both *au* and *yg-2* are blocked in phytochrome chromophore biosynthesis (Kendrick et al. 1994).

Thus, among the several candidates for mutations which block phytochrome chromophore synthesis, only the *hy1*, *hy2*, and *hy6* mutants have been clearly shown to be chromophore deficient (Parks and Quail 1991), and their sites of action remain to be determined. Identification of the precise steps blocked by these mutants will

contribute greatly to further elucidation of the details of the pathway for PΦB synthesis, and may provide confirmation of the enzymatic nature of certain poorly characterised steps. In addition, *hy1*, *hy2*, *hy6*, and *au* are all widely used as phytochrome deficient controls in genetic, physiological and biochemical studies (e.g. Neuhaus et al. 1993, Millar et al. 1995), and further knowledge about the site of action and potential secondary effects of such mutations will therefore assist in interpretation of this data.

During the course of the present work, a number of pea mutants were isolated as potential chromophore synthesis mutants. In this chapter the characterisation of one such mutant, *pcd1* is reported. Evidence is presented that the *pcd1* mutant is deficient in phytochrome chromophore synthesis and that this deficiency results from an inability of mutant plants to convert heme to BV IXα.

5.2. Results

5.2.1. Isolation and Inheritance of the *pcd1* Mutant

Pea mutant line S2-14 was selected for its yellow-green foliage and elongated internodes relative to parental WT cv. Solara (SOL) plants in an M_2 generation grown in the glasshouse under a natural photoperiod, by Dr Catherine Rameau at INRA, Versailles, France. In addition to pale foliage, mutant plants have elongated internodes, show reduced branching and flower slightly later than WT plants grown under the same conditions. The phenotypes of WT and S2-14 plants grown in continuous WL are shown in **Figure 5.1A**. A cross of S2-14 to SOL yielded an entirely WT F_1 and an F_2 segregation of 49 WTplants to 15 pale mutants ($\chi^2_{(3:1)} = 0.083$, $P>0.7$). A cross of S2-14 to the standard WT line, cv. Torsdag, returned an F_2 segregation of 82 WT plants: 28 pale mutants ($\chi^2_{(3:1)} = 0.012$, $P>0.9$). Perfect cosegregation of all characteristic aspects of the S2-14 mutant phenotype in accord with a 3:1 ratio indicated that this pleiotropic phenotype most probably results from a single recessive mutation. The mutation was named *pcd1* for *p*hytochrome *c*hromophore *d*eficient. **Figure 5.1B** shows the phenotype of the *pcd1* mutant after partial transfer to the cv. Torsdag background.

5.2.2. Photomorphogenesis and Phytochrome Content of *pcd1* Seedlings

Figure 5.2 shows the responses of *pcd1* to broad-band monochromatic light. The *pcd1* mutant was dramatically elongated under FR, appearing completely insensitive to FR both in terms of elongation and rate of development (**Figure 5.2A, B**). Because phyA is the predominant phytochrome mediating responses to continuous FR (**Chapter 4**; Reed et al. 1994, van Tuinen et al. 1995a), this result indicates that *pcd1* is strongly deficient in phyA activity. Dark-grown *pcd1* and WT plants were the same height (**Figure 5.2B**) and showed no other visible difference, indicating that expression of the *pcd1* phenotype is dependent on light, and therefore that the mutation is truly photomorphogenic in nature. A marked reduction in response of *pcd1* to R was also apparent, although the mutant did appear to retain some sensitivity to R (**Figure 5.2B**). This reduction in sensitivity to R is more severe in *pcd1* than in phyB-deficient *lv* mutants (**Chapter 3**) suggesting that *pcd1* shows strongly reduced activity of phyB and at least one other R-sensing phytochrome. In contrast, the sensitivity of *pcd1* to WL and to blue light was only slightly reduced compared to WT (**Figure 5.2**).

The response of *pcd1* to end-of-day (EOD)-FR treatment was also tested. **Figure 5.3** shows that WT plants respond strongly to EOD-FR treatment, which leads to increased internode elongation throughout the growth of the plant. In contrast, over the very early internodes, *pcd1* seedlings showed a greatly reduced reponse to EOD-FR. However, with increasing age, mutant seedlings gradually recovered the ability to respond, to the extent that newly produced internodes in 3-week-old *pcd1* plants showed the same relative response as those of WT plants. It has been shown that phyB is the principal phytochrome controlling the elongation response to EOD-FR, as phyB-deficient mutants of *Arabidopsis* (Nagatani et al. 1991), *Brassica* (Devlin et al. 1992), cucumber (López-Juez et al. 1992) and pea (Nagatani et al. 1990, **Chapter 3**) all lack this response. The response of the *pcd1* mutant is different from that of phyB-deficient *lv* mutants, which show no such recovery of EOD-FR response with age (Nagatani et al. 1990). This result shows that *pcd1* is strongly deficient in phyB activity at the seedling stage, but recovers phyB activity as the plant matures.

Mature WL-grown *pcd1* plants have a yellow-green phenotype (**Figure 5.1**) the severity of which varied considerably depending on the conditions under which the plants were grown. Under a number of different WL regimes, *pcd1* caused a reduction in Chl content and a large increase in the Chl *a*/ Chl *b* ratio. This was most pronounced in short photoperiods and least pronounced in plants grown in continuous light. Representative effects of *pcd1* on foliar Chl levels are shown in **Table 5.1.**

These aspects of the *pcd1* phenotype are also common to photomorphogenic mutants of *Arabidopsis* (*hy1*, *hy2*, and *hy6*) and tomato (*au* and *yg-2*). All of these mutants are severely deficient in spectrophotometrically detectable phytochrome (Koornneef et al. 1980, 1985, Chory et al. 1989a). The phytochrome content of *pcd1* was therefore examined. **Figure 5.4A** shows representative difference spectra for *in vivo* phytochrome phototransformation in standard samples of etiolated WTand *pcd1* tissue. Although samples from WTplants contained phytochrome routinely giving a signal of 40 to 50 units (one unit is $1 \times 10^{-3}\ \Delta\Delta A_{660\text{-}730\ nm}$), the signal in *pcd1* samples was below the detection limit of the spectrophotometer, which was about 0.3 units. Etiolated *pcd1* plants therefore contain less than 1% of the spectrophotometrically detectable phytochrome present in the wild-type.

The phytochrome apoprotein content of the *pcd1* mutant was also examined. Immunoblotting analysis of crude protein extracts showed that etiolated *pcd1* plants have normal levels of phyA apoprotein (**Figure 5.4B**). In addition, the depletion of phyA apoprotein seen in response to irradiation of WT seedlings with 4 h R was not observed for the *pcd1* mutant (**Figure 5.4B**). As the depletion of phyA after R is dependent on conversion to P_{fr}, this result suggests that phyA in the *pcd1* mutant does not undergo photoconversion. Both these results are consistent with the suggestion that phytochrome in *pcd1* plants lacks a chromophore.

5.2.3. Reconstitution of Spectrally Active *pcd1* Phytochrome

One possible explanation for the deficiency in phytochrome spectral activity in *pcd1* is that the apophytochrome has undergone modification that prevents assembly *in vivo*. To address this question, assembly of apophytochrome extracted from etiolated *pcd1* seedlings was attempted. To this end, phytochrome apoprotein from *pcd1* plants was partially purified in the presence of PCB. PCB is a structural analog of the native phytochrome chromophore precursor PΦB and derives from the light-harvesting chromoprotein C-phycocyanin. Previous reports have demonstrated that PCB can substitute for PΦB in the assembly of a spectrally and biologically active phytochrome holoprotein (Elich and Lagarias 1989, Parks and Quail 1991) and that this assembly is an autocatalytic process, requiring only apophytochrome and PCB (Lagarias and Lagarias 1989).

Figure 5.5 shows that the apophytochrome in *pcd1* extracts assembled with PCB to give a spectrally active phytochrome, when PCB was included in the extraction buffer. The difference spectrum for this holophytochrome had absorption peaks in the

R (652 nm) and FR (716 nm) which were blue-shifted by 16 and 14 nm, respectively, relative to the corresponding peaks for phytochrome in WT samples. These are very close to the peak values previously reported for a pea apophytochrome A-PCB adduct (Deforce et al. 1993). Typically, 65-80% of WT spectral activity was recovered in *pcd1* extracts incubated with PCB (based on equivalent tissue fresh weight). Apophytochrome that was extracted from *pcd1* tissue in the absence of PCB, but subsequently assembled with PCB added to the partially purified extract, resulted in a holoprotein with spectral characteristics identical to those shown in **Figure 5.5.** However, yields were considerably lower **(Table 5.2)**, indicating that the apoprotein was not stable during the extraction procedure. These results clearly show that the loss of spectral activity of the phytochrome in *pcd1* is due to a deficiency of the endogenous chromophore, rather than reflecting incompetence of the apoprotein to bind chromophore.

5.2.4. Biliverdin Restores Phytochrome Spectral Activity to *pcd1 In Vivo*

The possibility was then examined that the *pcd1* mutation might lead in some other way to the failure or prevention of assembly *in vivo*. To test this hypothesis, recovery of holophytochrome was attempted *in vivo* by feeding chromophore precursors to intact seedlings. It has previously been shown that both exogenously supplied PCB and BV can restore a WT level of spectrally active phytochrome and a WT light-grown phenotype in the chromophore-deficient mutant *hy1* of *Arabidopsis* (Parks and Quail 1991). The possibility that the *pcd1* mutant might be rescued in a similar manner was tested by supplying these chromophore precursors to germinating *pcd1* seeds. Although several methods of supplying PCB and BV to germinating seedlings were tested, including inbibition of the seeds and/or saturation of the growing medium with bilin solution, no restoration of light-induced inhibition of stem elongation or phytochrome spectral activity was seen (data not shown). However, because the ineffectiveness of these compounds could have resulted from problems with uptake from the growing medium, an alternative method was employed, in which excised tissue segments were floated in a solution of BV (Elich and Lagarias 1987, Elich et al. 1989). Because pea shoot tissue treated in this way retained substantial amounts of BV that could not be washed out, it was impossible to obtain valid *in vivo* measurements of phytochrome. Phytochrome was therefore partially purified phytochrome from BV treated explants and spectral activity was measured *in vitro*. PCB was not used because the possibility of assembly during the extraction procedure could not be excluded.

The difference spectrum of the newly synthesised holophytochrome is shown in **Figure 5.6**. This spectrum shows no substantial deviation from the spectrum of phytochrome extracted from WT tissue either in shape or in wavelength of the ΔA peaks (666 and 732 nm for both samples). Feeding of the natural BV isomer, BV IXα, gave an identical result. The amount of holoprotein in *pcd1* after BV incubation was about 27% of that found in WT (based on equivalent fresh weight), a recovery that compares favorably with the value reported for gabaculine-treated oat coleoptiles (34%) using a higher concentration of BV (500 μM, Elich and Lagarias 1987).

Because BV itself does not assemble in vitro with apophytochrome to give a spectrally active holoprotein (Li and Lagarias 1992), the recovery of spectral activity in *pcd1* indicates that BV has been converted to PΦB and that assembly of holophytochrome has occurred in vivo. This result therefore shows that apophytochrome synthesis and assembly are normal in *pcd1* and that the mutant is deficient in phytochrome chromophore synthesis. In addition, the recovery of signal obtained with BV suggests that the block in chromophore synthesis imposed by *pcd1* most probably occurs prior to BV.

5.2.5. Heme Content of *pcd1*

As shown in **Figure 1.5**, the immediate precursor of BV IXα in PΦB synthesis is thought to be heme. Because the *pcd1* mutation is sufficiently severe to prevent detectable PΦB synthesis, a block in the pathway prior to heme would also be expected to result in reduced heme levels. Previous attempts to recover phytochrome spectral activity from chromophore depleted oat explants incubated with heme were reported to be unsuccessful (Elich et al. 1989) and a similar experiment was therefore not attempted. Instead, to address whether the *pcd1* mutation might have its effect prior to heme formation, total noncovalently bound heme (as an estimate of total cellular heme) was quantitated in WTand *pcd1* seedlings. As shown in **Table 5.2**, no significant difference was found in the level of noncovalently bound heme in WT and *pcd1* seedlings. Hemes are also a component of all cytochromes, and are therefore essential to the basic cellular functions of photosynthesis and respiration. Heme deficiency might therefore be expected to seriously compromise the general vigor of the plant. However, consistent with the heme quantitation data, *pcd1* plants are generally healthy plants and are not noticeably less vigorous than WT plants (see **Figure 5.1**). These results, when taken together with the fact that *pcd1* plants are able to utilise BV (**Figure 5.6**), provide indirect evidence that the *pcd1* mutation may prevent the conversion of heme to BV IXα.

5.2.6. Phytochromobilin Synthesis in *pcd1* Plastids

To specifically address the question of whether the *pcd1* mutant is unable to convert heme to BV IXα and PΦB, a recently developed assay system for the detection of PΦB was employed (Terry et al. 1995). Isolated etioplasts were incubated with heme in the presence of an NADPH regenerating system and the products were analysed by reverse phase HPLC. **Figure 5.7** shows that WT plastids converted heme to a major product with a retention time of approximately 11 min (**trace c**). The retention time of this peak is consistent with its identification as either BV IXα or 3*Z*-PΦB, which coelute in this solvent system (Terry et al. 1995). To identify this peak unequivocally the products were reexamined using a modified HPLC solvent system that can resolve BV IXα from 3*Z*-PΦB (Terry et al. 1995). This HPLC trace is shown in **Figure 5.8A.** Under these conditions the original single peak resolved into two (labeled *a* and *b*, **Figure 5.8A**). Coinjection studies with authentic BV IXα and 3*Z*-PΦB samples provisonally identified peaks *a* and *b* (**Figure 5.8A**) as 3*Z*-PΦB and BV IXα, respectively (MJ Terry pers. comm.). The low yield of peak *b* precluded more rigorous identification, but peak *a* was purified and analysed further.

Figure 5.8B shows an absorption spectrum of peak *a* with BV IXα also shown for comparison. The spectrum of peak *a* is red-shifted in comparison to BV IXα, consistent with its identification as 3*Z*-PΦB (Terry et al. 1995). For final verification of the identity of peak *a*, an assembly reaction was attempted with partially purified apophytochrome derived from the *pcd1* mutant. It has previously been demonstrated that 3*Z*-PΦB will assemble with apophytochrome, although it is not clear whether isomerisation to the 3*E*- isomer is required for the assembly to proceed (Terry et al. 1995). As shown in **Figure 5.8C**, assembly of peak *a* with pea apophytochrome results in a holophytochrome with a difference spectrum that is almost identical to the WT pea phytochrome under these conditions (**Figures 5.5** and **4.6**). This result confirms that the major product from the incubation of WT plastids with heme is 3*Z*-PΦB.

Incubation of WT plastids with heme also resulted in the synthesis of a product with a retention time of about 19 min (**Figure 5.7, trace c**). This was identified as 3*E*-PΦB by coinjection with the authentic standard (MJ Terry, pers. comm.). Because the major product was 3*Z*-PΦB, the additional synthesis of 3*E*-PΦB would be expected (Terry et al. 1995). Incubation of WT plastids in the absence of heme also resulted in the synthesis of 3*Z*-PΦB, though the yield was greatly reduced (**Figure 5.7, trace a**). This may reflect synthesis from endogeneous chromophore precursors. Incubations of heme without plastids resulted in some coupled oxidation of the heme leading to the

appearance of a number of small peaks, one of which was identified as BV IXα (data not shown). When plastids isolated from *pcd1* seedlings were incubated with heme, the 3*Z*-PΦB peak was almost completely absent and no 3*E*-PΦB was detected (**Figure 5.7, trace d**). No peaks were discernible from incubations of *pcd1* plastids in the absence of heme (**trace b**). These results clearly demonstrate that isolated *pcd1* plastids are unable to convert heme to 3*Z*-PΦB.

To confirm that *pcd1* can synthesise PΦB from BV IXα and that isolated plastids from *pcd1* are functionally active, the same plastid preparations were incubated with BV IXα (**Figure 5.9**). The metabolism of BV IXα to PΦB has previously been well characterised using oat etioplasts (Terry et al. 1995). As shown in **Figure 5.9**, both WTand *pcd1* plastids converted BV IXα to 3*Z*- and 3*E*-PΦB, with the products being confirmed by coinjection studies and absorption spectroscopy (MJ Terry, pers. comm.). This result confirms that the conversion of BV IXα to 3*Z*- and 3*E*-PΦB is normal in *pcd1* and that the mutant is specifically deficient in the synthesis of BV IXα from heme.

5.3. Discussion

Phytochromobilin Synthesis in the *pcd1* Mutant

Heme has been proposed to be the precursor of BV IXα in the biosynthesis of the phytochrome chromophore. The proposal is based on analogy with the pathway for phycobilin biosynthesis in the red alga *Cyanidium caldarium* (Beale 1993, Terry et al. 1993b) and is supported by two pieces of experimental evidence. Isolated cucumber etioplasts can use heme, but not Mg-protoporphyrin, to synthesise PΦB detected by assembly to apophytochrome (Terry et al. 1993b). Secondly, application of an inhibitor of ferrochelatase, which is required for heme synthesis (see **Figure 1.5**), results in a reduction in spectrophotometrically detectable phytochrome in embryonic axes of pea (Konomi et al. 1993). In our assays, the major product following the incubation of heme with WT pea plastids was 3*Z*-PΦB, whereas BV IXα was only present in trace amounts (**Figures 5.7, 5.8**). Plastids from *pcd1* seedlings were unable to synthesise 3*Z*-PΦB from heme (**Figure 5.7**), although the same plastid preparation could convert BV IXα to 3*Z*-PΦB (**Figure 5.9**). Together with the demonstration that *pcd1* seedlings are not heme deficient (**Table 5.2**) and that BV can restore holophytochrome to *pcd1 in vivo* (**Figure 5.6**), these results clearly demonstrate that *pcd1* is unable to convert heme to BV IXα. These experiments therefore confirm the

proposal that heme is an intermediate in the synthesis of the phytochrome chromophore.

We consider it probable that the *pcd1* mutation lies in the catalytic subunit of the enzyme accomplishing the conversion of heme to BV IXα. Unfortunately, there are no molecular probes available for this enzyme and it was not possible to test this hypothesis directly. The enzymatic conversion of heme to BV IXα in higher plants is thought to be accomplished by the enzyme heme oxygenase as it is in mammals and red algae (Maines 1988, Cornejo and Beale 1988, Beale 1993). This activity has not previously been measured in higher plants, in part due to the problem of distinguishing facile, non-enzymatic coupled oxidation of heme from the enzyme-catalysed reaction (Terry et al. 1993b). The absence of this reaction in *pcd1* provides direct evidence for this step being enzyme-catalysed in higher plants. However, since the reaction proceeds to 3Z-PΦB, no further characterisation has been performed and the nature of the reaction from heme to BV IXα remains unknown at present. The reason that 3Z-PΦB, and not BV IXα, is the major product following the incubation of heme with isolated plastids is also unknown. However, there is no absolute requirement for the presence of PΦB synthase since, in mutants unable to synthesise 3Z-PΦB from BV IXα, incubation of heme results in BV IXα accumulation (see **Chapter 6,** Terry and Kendrick 1996).

Phenotype of the *pcd1* Mutant

Most of the observed aspects of the *pcd1* phenotype may be readily explained in terms of the deficiency in active phytochrome which results from the demonstrated block in PΦB synthesis. The mutant is clearly lacking in both phyA and phyB responses at the seedling stage, indicating that the PΦB deficiency results in a reduction in the level of activity of at least two and possibly all phytochromes in pea. One notable aspect of the *pcd1* phenotype is the gradual recovery of the phyB-mediated EOD-FR response. This suggests that more active phyB is present in the mature tissues of *pcd1* plants and thus implies that more PΦB has been synthesised. This is likely to result from leakiness of the *pcd1* mutation, although it might also reflect the presence of a *PCD1* homolog, expressed at higher levels in mature tissue. The location of the *pcd1* lesion after the branch point for chlorophyll and PΦB synthesis shows that the pale phenotype of the mutant does not result from a direct impairment of Chl synthesis. However, phytochrome has well-documented effects on plastid development and on the synthesis of Chl (Kasemir 1983) and Chl *a*/*b* -binding proteins (Batschauer et al. 1994), and it is likely that one or more of these effects are sufficient to account for the

pale phenotype. In addition, negative feedback effects of free heme on aminolevulinic acid synthesis have been proposed (Chereskin and Castelfranco 1982). Although there was no difference in noncovalently bound heme (**Table 5.2**), it is possible that an accumulation of free heme in the *pcd1* mutant might occur, and this could also contribute to the chlorotic phenotype.

Relationship of *pcd1* to Other Mutants

Mutants similar in phenotype to *pcd1* have been identified in several other species. Mutants *au* and *yg*-2 of tomato, *hy1*, *hy2*, and *hy6* of *Arabidopsis*, and *pew 1* and *pew2* of *Nicotiana plumbaginifolia* are all pale, are elongated in FR and R, and have reduced levels of spectrally active phytochrome (Koornneef et al. 1980, 1985, Chory et al. 1989a, Kraepiel et al. 1994). These mutants are all considered to be chromophore-deficient mutants, although this has been conclusively proven only for the *hy* mutants (Parks and Quail 1991, Nagatani et al. 1993). Although pale, the *au* and *yg*-2 mutants are relatively healthy and vigorous suggesting that these plants, like *pcd1*, are not deficient in heme (Kendrick et al. 1994). The lesions in *au* and *yg*-2 are therefore also likely to affect steps after the formation of heme. Again, although the *hy1*, *hy2*, and *hy6* mutants have reduced Chl levels (Chory et al. 1989a) and would be expected to have impaired photosynthetic ability, all three mutants are generally healthy. These mutants are therefore also unlikely to be deficient in heme, suggesting that they may be blocked after heme formation as well. Recent evidence from heme and BV IXα metabolism studies in *au* and *yg*-2 has confirmed that both mutants are in fact blocked in PΦB synthesis after this point (Terry and Kendrick 1996). The *hy1* mutant is clearly rescued by BV (Parks and Quail 1991) and it therefore seems probable that this mutation may affect the same step as *pcd1*, preventing the conversion of heme to BV IXα . By the same reasoning, *hy2* has also been suggested to lie before BV (Parks and Quail 1991). However, the incomplete rescue of *hy2* on BV (Parks and Quail 1991) may indicate that the mutation is in fact after BV IXα. Such a mutation, if leaky, might still allow synthesis of significant amounts of PΦB in the presence of BV at a sufficiently high concentration. However, further investigation of the pathway for PΦB synthesis in *hy1* and *hy2* will clearly be necessary to address these questions.

The results presented in this study clearly identify the biochemical basis of the *pcd1* phenotype. They conclusively demonstrate that the phenotype results from impairment of a specific, committed step in phytochrome chromophore biosynthesis. Furthermore, they establish that heme is an intermediate in the synthesis of the phytochrome chromophore. The feasibility of addressing this kind of biochemical

problem in pea highlights an advantage of this species for a mutant-based approach to understanding the function of phytochrome in higher plants. The present study should greatly assist in the investigation of similar mutants in other species which are less amenable to biochemical investigation, and should ultimately lead to a better understanding of phytochrome chromophore synthesis and its role in plant photomorphogenesis.

5.4. Methods

5.4.1. Plant Material and Growth Conditions

The original *pcd1* mutant line S2-14 was derived from cv. Solara (genotype *le af i*) at INRA Station de Genetique et d'Amelioration des Plantes in Versailles, France. Imbibed seed of cultivar Solara were mutagenised by soaking in 4 mM ethyl methanesulfonate (EMS) for 4 h at room temperature. The S2-14 mutant was identified in an M_2 population grown in a glasshouse under natural daylength conditions. The mutant line was bred by single plant selection for 3 generations before backcrossing to cultivar Solara. Unless otherwise indicated, all *pcd1* material used in this study derived from *pcd1* segregates in the F_4 and F_5 generations of this cross. All plants were grown in drained, water-saturated vermiculite (experiments in **Figures 5.4-5.10**) or in standard Hobart pea potting mix. The initial screen under monochromatic light and plants for chlorophyll (Chl) extractions were grown in growth cabinets at 20°C, using standard Hobart light sources. Light sources for the EOD-FR experiment (**Figure 5.3**) were identical to those described by Weller and Reid (1993). Plants for all other experiments were grown in growth cabinets at 25°C. The standard RIKEN R source was used to deplete phytochrome in the immunoblotting experiment (**Figure 5.4B**). Standard green safety light was used for manipulation of etiolated plants and during all biochemical experiments.

5.4.2. Protein Extraction and Immunoblotting

Crude protein extracts were prepared from dark-grown or R-treated seedlings and subjected to SDS-PAGE and immunoblotting as described in **Chapter 3.** Protein was quantitated by the method of Bradford (1976) using Bio-Rad protein assay reagent and BSA as a standard.

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5.4.3. Reagent Preparation

BV IXα was obtained from Porphyrin Products, Inc. (Logan, UT, USA) and further purified by C18 reverse phase HPLC using a Shimadzu LC10AS liquid chromatograph system and a Supelcosil LC-18 ODS column (4.6 x 25 cm, 5 mm particle diameter; Supelco Inc., Bellefonte, PA, USA). The mobile phase consisted of ethanol-acetone-water-acetic acid (48:34:17:1 [v/v], mobile phase A) with a flow rate of 1.5 mL/min and the column eluate was monitored at 380 nm. HPLC-purified BV IXα was concentrated by diluting 4 times with 0.1% (v/v) trifluoroacetic acid (TFA) and applying to a C18 Bond Elut column (1 mL; Varian, Harbor City, CA, USA). After washing with 0.1% TFA, BV IXα was eluted with acetonitrile/0.1% TFA (3:2 [v/v]) and dried in vacuo. For BV feeding experiments to apical segments, BV (a mixture of isomers) was obtained from Sigma. Phycocyanobilin (PCB) was purified from *Spirulina platensis* as described previously (Terry et al. 1993a). The sample contained >90% 3*E*-PCB when analysed by HPLC. 3*E*-Phytochromobilin (3*E*-PΦB) was a gift from J.C. Lagarias (University of California, Davis, CA, USA). 3*Z*-PΦB was purified by HPLC following conversion of BV IXα to PΦB by isolated pea etioplasts (see below; also Terry et al. 1995). HPLC conditions were identical to those for BV IXα purification except that the mobile phase was changed to ethanol-acetone-100 mM formic acid (25:65:10 [v/v], mobile phase B) to increase the resolution between BV IXα and 3*Z*-PΦB (Terry et al. 1995). All bilins were prepared as 1 mM stock solutions in DMSO using the following molar absorption coefficients: 66,200 M cm^{-1} at 377 nm for BV IXα (McDonagh and Palma 1980), 47,900 M cm^{-1} at 374 nm for 3*E*-PCB (Cole et al. 1967) and 64,570 M cm^{-1} at 386 nm, and 38,020 M cm^{-1} at 382 nm for 3*E*-PΦB and 3*Z*-PΦB, respectively (Weller and Gossauer 1980). Absorption spectrophotometry of bilin and heme samples was performed using a Hitachi U-3410 spectrophotometer (Hitachi, Tokyo, Japan). A stock solution of heme was prepared by dissolving hemin chloride (Sigma) in 0.1 M NaOH and adjusting to pH 7.7 with 1N HCl. The final heme concentration was 1 mM. Unless otherwise stated all chemicals and reagents were purchased from Sigma, Kanto Chemical Co. (Tokyo, Japan) and Nakalai Tesque (Kyoto, Japan).

5.4.4. Phytochrome Assembly *In Vivo*

For experiments involving feeding of BV to floating shoot tips (**Figure 5.6**), 40-50 apical segments (2-cm long) from 5-day-old seedlings (about 3 g fresh weight) were harvested in green safe-light under buffer (15 mM Hepes, NaOH, pH 7.4) and transferred to Petri dishes containing 300 μM BV IXα in the same buffer. The apical

segments were then cut into smaller segments, 3 to 4 mm in length, and floated with gentle shaking for 6 h in darkness. After floating, the tissue was rinsed several times, blotted dry, weighed, frozen in liquid nitrogen and stored at -80°C until phytochrome extraction.

5.4.5. Phytochrome Extraction and Assembly *In Vitro*

For each extraction, 40-50 apical segments (2-cm long) from 5-day-old etiolated seedlings (about 3 g fresh weight) were harvested and homogenised in liquid nitrogen. The homogenate was suspended in a 3/1 (v/w) volume of extraction buffer (50 mM Tris, 100 mM ammonium sulfate, 25% [v/v] ethylene glycol, 1mM EDTA, 2 mM phenylmethanesulfonyl fluoride (PMSF), 10 mM diethyldithiocarbamate, 142 mM β-mercaptoethanol, 4 mM cysteine, 2 μg/mL leupeptin, 2 μg/mL aprotinin, and 1 μg/mL pepstatin, adjusted with HCl to pH 8.3 at 5°C) and extracted with gentle stirring for 30 min at 4°C in the presence or absence of 3 μM PCB. After centrifugation for 30 min at 200 000*g*, saturated ammonium sulfate solution (0.725:1, [v/v]) was added to the supernatant, and the extract was gently stirred for an additional 30 min. The ammonium sulfate pellet was collected by centrifugation (30 min at 30 000*g*) and resuspended in 1 mL TEGE buffer (25 mM Tris, 2 mM EDTA, 25% ethylene glycol [v/v], 2 mM PMSF, 1 mM DTT, and 2 μg/mL leupeptin, adjusted with HCl to pH 7.8 at 5°C). The extract was then clarified by centrifugation (15 min at 200 000*g*) prior to spectrophotometric assay. Extracts were maintained at or below 4°C throughout the extraction. For assembly experiments with extracted apophytochrome samples, the bilins were added to the clarified extract and incubated on ice for 30 min.

5.4.6. Spectrophotometric Assay for Phytochrome

All spectrophotometric determinations were made using a recording difference spectrophotometer (model 3410, Hitachi Ltd. Tokyo, Japan). Standard samples for *in vivo* spectrophotometric phytochrome determinations were prepared as described in **Chapter 2**. All samples and extracts were kept at 4°C during measurements. Actinic beams for phytochrome photoconversion were obtained by filtering light from a xenon lamp (150 W, Ushio Inc., Tokyo, Japan) through a 658 nm or a 748 nm interference filter (Vacuum Optics Corp., Tokyo, Japan). Phytochrome was photoconverted by exposure to 60 sec R and 90 sec FR (*in vivo* determinations) or 180 sec R and 300 sec FR (*in vitro*), irradiations sufficient to induce essentially full photoconversion as determined by kinetic measurements.

5.4.7. Assays for Phytochromobilin Synthesis

PΦB synthesis from heme and BV IXα was assayed in isolated pea etioplasts essentially as described previously for PΦB synthase assays using oat etioplasts (Terry et al. 1995). Etioplasts were isolated by differential centrifugation from 8-day-old dark-grown seedlings as described by Terry and Lagarias (1991) with the following modifications. PVP (0.5% [w/v]) was added to the homogenisation medium, and the final crude plastid pellet was washed once with assay buffer stock (20 mM TES/10 mM Hepes, NaOH pH 7.7, containing 500 mM sorbitol) before use. PΦB synthesis assays were performed in 20 mM TES/10 mM Hepes/NaOH buffer pH 7.7 containing 500 mM sorbitol, 1 mM PMSF, 0.5 mM DTT, 2 μM leupeptin, 3000 units mL^{-1} catalase, and an NADPH regenerating system (1.2 mM $NADP^+$, 10 mM glucose 6-phosphate and 1.5 units mL^{-1} glucose 6-phosphate dehydrogenase). The reaction was initiated by the addition of either heme (10 μM final concentration) or BV IXα (8 μM). For the assay with BV IXα, an argon treatment was used to deplete oxygen. The reaction volume was 0.5 mL and reaction mixtures were incubated in the dark at 28°C with shaking. Following a 3-h incubation, samples were partially purified using a Bond Elut column (see above) as described previously (Terry et al. 1995). The elution volume was 1 mL. HPLC analysis of bilins was performed as described above using mobile phase A (**Figures 5.7, 5.9**) or B (**Figure 5.8**).

5.4.8. Tetrapyrrole Quantitation

Total noncovalently bound heme was extracted from the top 2 to 3 cm of 8-day-old dark-grown seedlings, essentially as described by Thomas and Weinstein (1990). Harvested tissue (5 g fresh weight) was homogenised in 20 mL of cold 90% (v/v) acetone containing 10 mM NH_4OH. After centrifugation at 4 000*g* for 2 min, the pellet was washed twice more in the same volume. Noncovalently bound hemes were extracted with 2 x 10 mL of cold 80% (v/v) acetone containing 5% (v/v) HCl. The heme extracts were pooled, transferred to chloroform-butanol (2:1, [v/v]) and washed twice with water. The heme was then concentrated by application to a DEAE-Sepharose column (Bond Elut DEA, 3 mL; Varian) with an equal volume of 95% (v/v) ethanol. Following washes with chloroform-butanol (2:1) and 95% ethanol, the heme was eluted in 2 mL ethanol-acetic acid-water (70:17:13, [v/v]). The concentration was determined by absorption spectroscopy (see above) using the molar absorption coefficient of 144 000 M cm^{-1} at 398 nm calculated for air-oxidised protoheme in this solvent (Weinstein and Beale 1983).

Table 5.1. Chl content and Chl *a* / Chl *b* ratio of the *pcd1* mutant

Photo-period (hr)	Genotype	Chl Content (mg g fresh weight^{-1}) [a]			Chl *a*:*b*
		Chl *a*	Chl *b*	Total Chl	
24	WT [b]	1.67±0.12	0.52±0.04	2.19±0.16	3.2
	pcd1	1.41±0.16	0.17±0.25	1.58±0.18	8.4
8	WT	1.50±0.12	0.38±0.03	1.88±0.14	3.9
	pcd1	0.25±0.01	0.03±0.01	0.28±0.01	12.0

[a] Values are expressed as the mean (±SE) of 6 samples. Tissue samples were taken from stipules at node six of four-week-old plants. Wild-type cv. Solara has no true leaflets.

[b] Wild-type

Table 5.2. Recovery efficiencies for *in vitro* and *in vivo* assembly experiments

Genotype	Treatment	$DDA_{660\text{-}800\ nm}$ (g fresh weight)$^{-1}$	% of WT[a]
WT		13.01 ± 0.45	
pcd1	+ PCB before[b]	9.60 ± 0.40	73.3 ± 7.1
pcd1	+ PCB after[c]	3.45 ± 0.45	22.9 ± 1.3
WT		8.60 ± 0.76	
WT	+ BV	8.42 ± 0.93	
pcd1	+ BV[d]	2.37 ± 0.72	26.8 ± 6.5

Values are expressed as mean ± SE for 4 (PCB assembly) or 3 replicates (BV feeding).

[a]Estimates of recovery percentage are based on the assumption that the absorption coefficients of the PCB adduct and native phytochrome are approximately equal. The value for *pcd1*+BV is calculated relative to WT+BV samples.

[b]assembly during extraction; 3 μM PCB included in extraction buffer.

[c]assembly after extraction; 3 μM PCB added to clarified extract.

[d]BV IXa was used for a single replicate. In the remaining replicates crude BV was used.

Table 5.3. Quantitation of Noncovalently Bound Heme in *pcd1* Seedlings

Genotype	Heme [a]	
	nmol (g fresh weight)$^{-1}$	pmol (seedling)$^{-1}$
WT [b]	0.56±0.15	67.2±16.0
pcd1	0.55±0.12	70.8±14.3

[a] Values are expressed as mean ± SE of 4 replicate measurements.

[b] Wild-type

Experiment performed by M. Terry

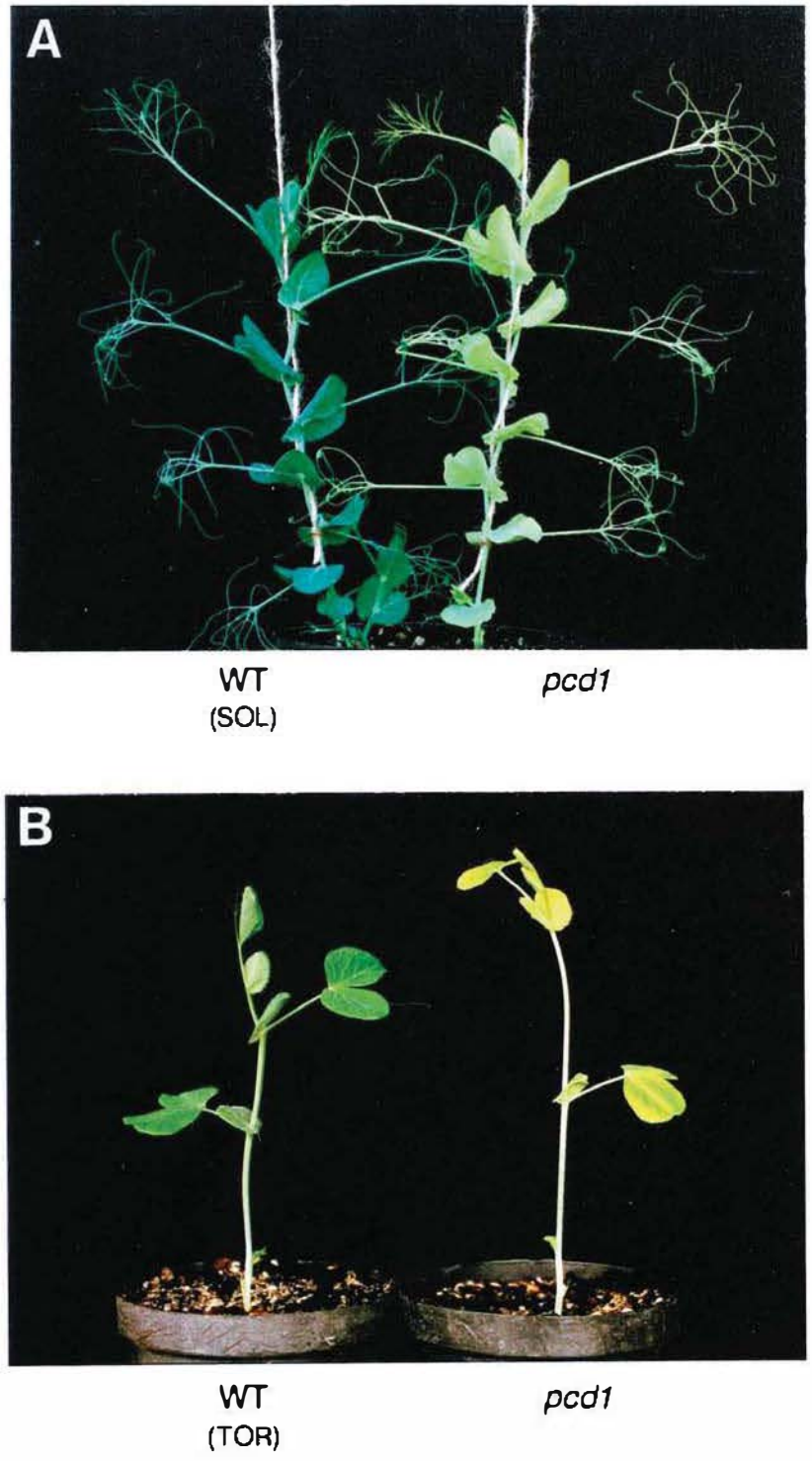

Figure 5.1. Phenotype of the *pcd1* mutant. **A.** The original *pcd1* mutant line S2-14 is shown with its progenitor, wild-type (WT) cv. Solara (SOL), which carries Mendel's dwarfing *le* mutation and the *afila* mutation (homeotic conversion of leaflets to tendrils). **B.** WT cv. Torsdag (TOR) is shown, together with a representative *pcd1* mutant segregate from the F_2 of the second back-cross of S2-14 to TOR. Growing conditions; standard WL, 150 μmol $m^{-2}sec^{-1}$, 20°C, for 30 d(A) or 11 d (B).

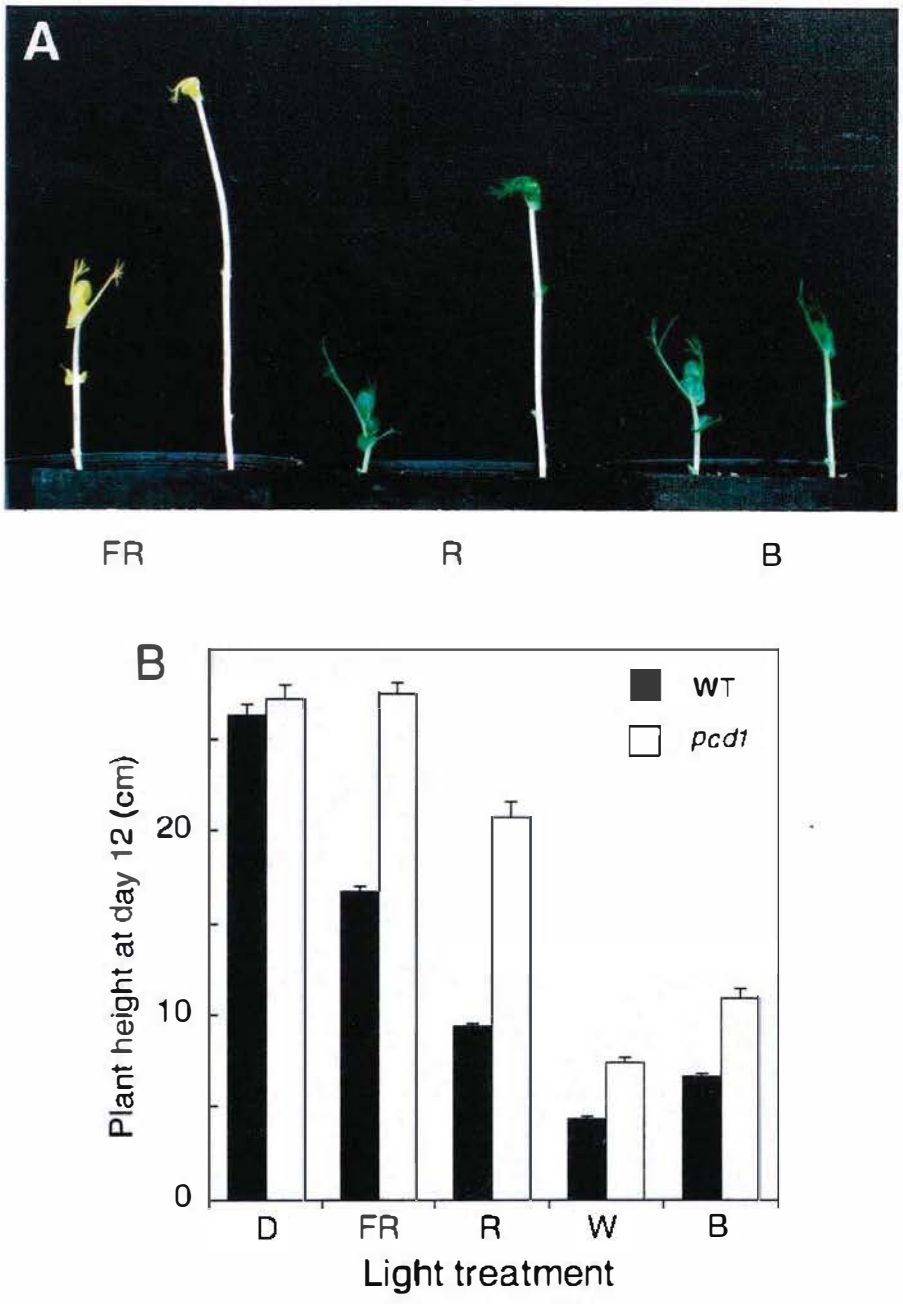

Figure 5.2. Phenotype of the *pcd1* mutant grown under monochromatic light. Seedlings were grown in the dark or under FR (8 μmol m^{-2} sec^{-1}), R (20 μmol m^{-2} sec^{-1}), WFL (150 μmol m^{-2} sec^{-1}) or B (10 μmol m^{-2} sec^{-1}), at 20°C. **A.** Representative WT cv. Solara (left of each pair) and *pcd1* seedlings. **B.** Total plant height. Error bars represent SE, n=12 -15.

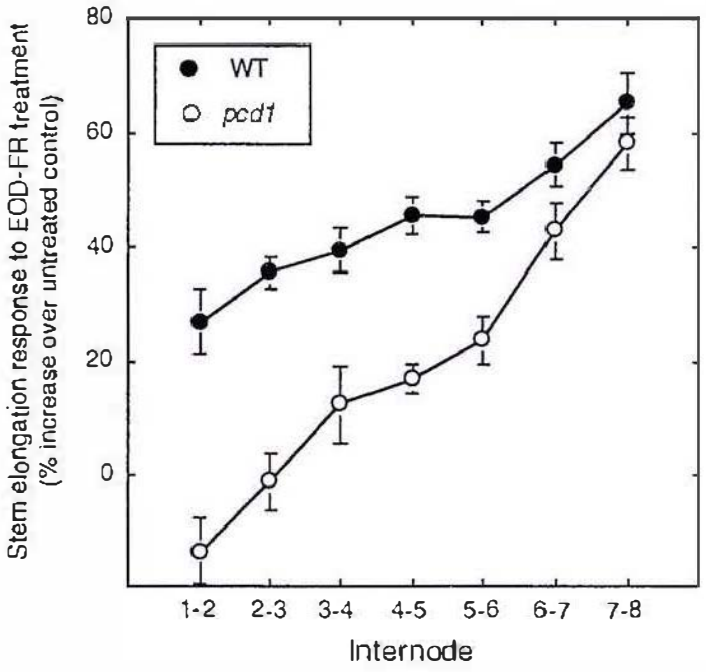

Figure 5.3. Elongation response of *pcd1* to EOD-FR treatment. Plants were grown for 3 weeks at 20°C in a 12-h light/dark cycle with or without a 20 min EOD-FR treatment (8 μmol m^{-2} sec^{-1}). The absolute increase in length in response to EOD-FR was 70±4 mm for the wild-type and 48±5 mm for *pcd1* over internodes 1 to 8. Error bars represent SE, n=12-15.

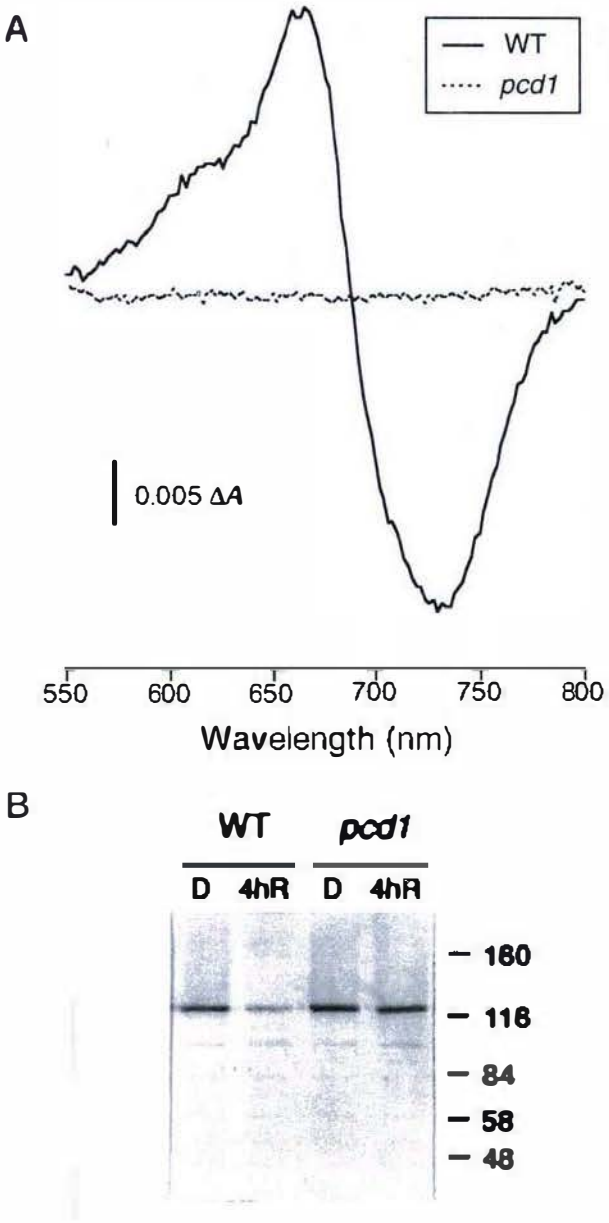

Figure 5.4. Phytochrome content of *pcd1* seedlings. **A.** Difference spectrum for *in vivo* phytochrome phototransformation (P_{fr}-P_r) in etiolated WT cv. Solara and *pcd1* seedlings. **B.** PHYA apoprotein content in crude protein extracts from WT and *pcd1* seedlings grown in complete darkness (D) or given a 4-h R treatment (17 μmol m^{-2} sec^{-1}) prior to harvest. The positions and molecular masses (kD) of prestained markers (Sigma) are indicated. Lanes were loaded on an equivalent fresh weight basis. Phytochrome was detected using the anti-pea PHYA monoclonal antibody mAP5 (Nagatani et al., 1984).

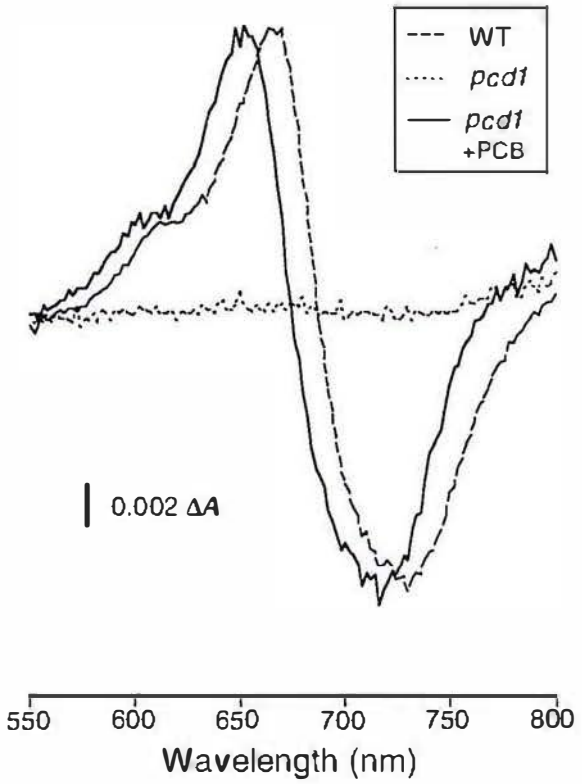

Figure 5.5. *In vitro* assembly of *pcd1* phytochrome apoprotein with phycocyanobilin (PCB). Difference spectra are shown for phytochrome (P_{fr}-P_r) in extracts from WT cv. Solara, *pcd1*, and *pcd1* tissue extracted in the presence of 3 μM PCB (*pcd1*+PCB). The WT spectrum has been scaled down for ease of comparison and the scale bar applies to *pcd1* and *pcd1*+PCB spectra only.

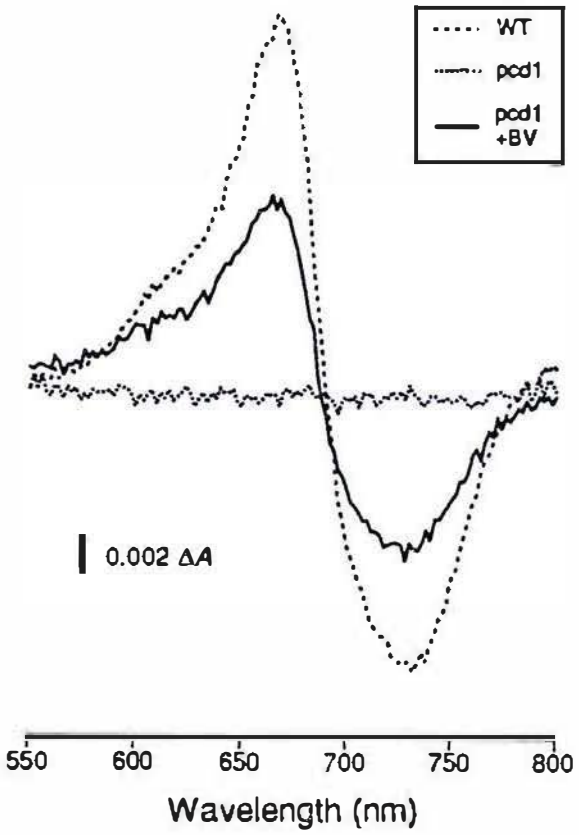

Figure 5.6. Recovery of phytochrome spectral activity in *pcd1* extracts incubated with BV. Difference spectra (P_{fr}-P_r) for phytochrome in extracts from WT cv. Solara, *pcd1*, and BV-incubated *pcd1* tissue (*pcd1*+BV). Apical explant sections were floated in 300 μM BV or buffer alone for 6h at room temperature. The wild-type spectrum has been scaled down for ease of comparison. The scale bar applies to *pcd1* and *pcd1*+BV spectra only.

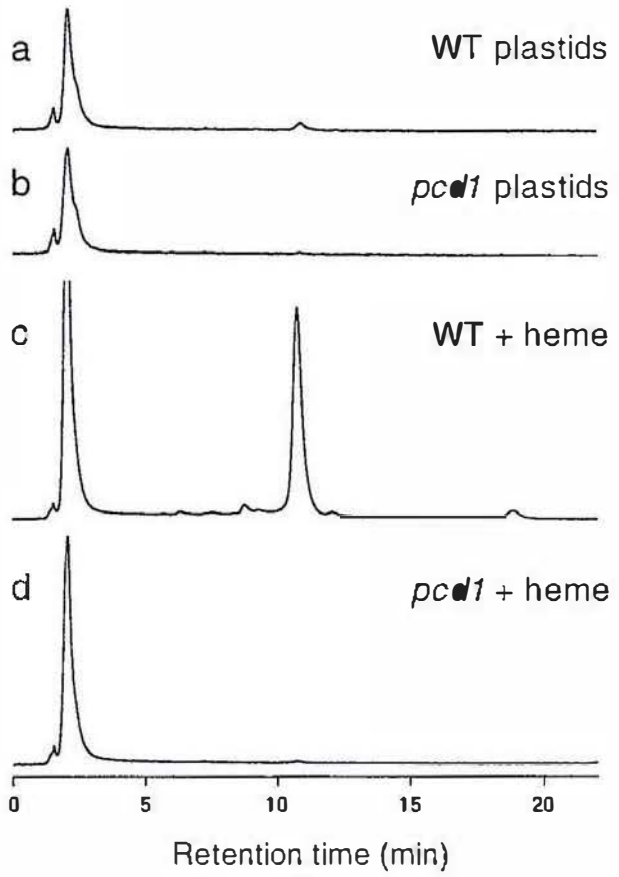

Figure 5.7. HPLC analysis of heme metabolism in WT and *pcd1* plastids. Plastids from etiolated seedlings of WT cv. Solara (traces a and c) and *pcd1* (band d) were incubated in the presence (c and d) or absence (a and b) of 10 μM heme. Incubations were for 3h at 28°C and also included an NADPH regenerating system. The final protein concentrations were 1.17 mg/ ml for WT and 1.16 mg/ ml for *pcd1*. Products were analyzed by reverse phase HPLC using a solvent system of ethanol-acetone-water-acetic acid (48:34:17:1, [v/ v]) and absorbance was monitored at 380 nm. The absorbance scale for samples containing plastids only (traces a and b) is half that of the samples from incubations with heme (c and d). Experiment performed by M. Terry.

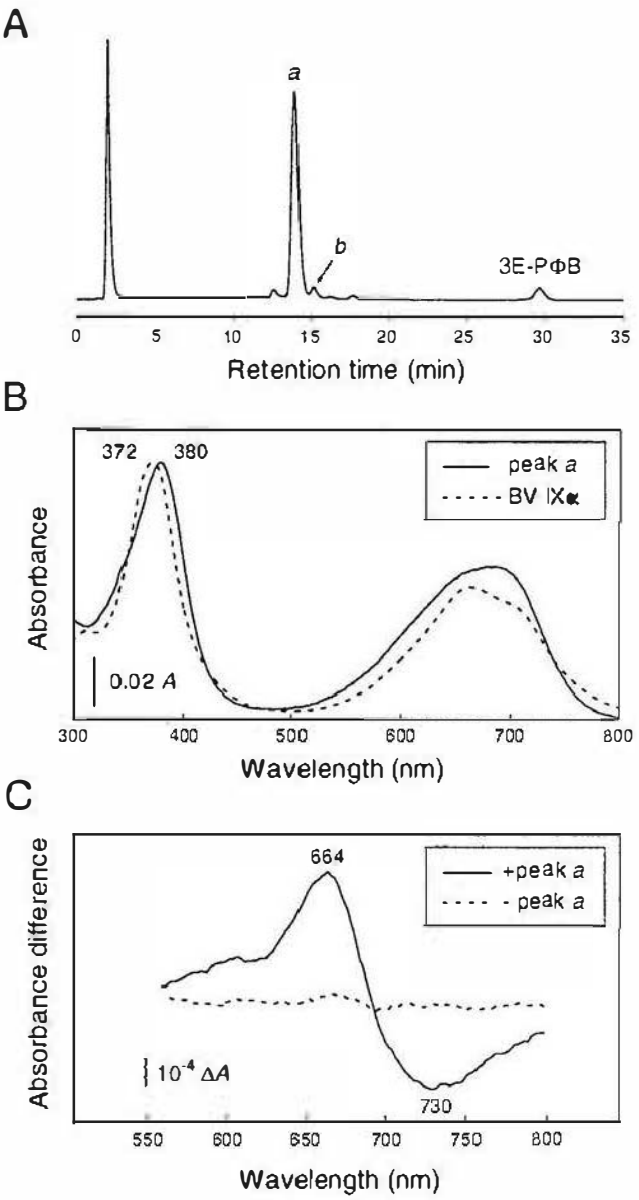

Figure 5.8. Analysis of the major product from the incubation of heme with WT plastids. **A.** Reverse phase HPLC analysis of the products formed from incubation of 10 μM heme with WT cv. Solara etioplasts. Incubations were for 3 h at 28°C and included an NADPH regenerating system. The final protein concentration was 0.84 mg/ ml. The solvent system used was ethanol-acetone-100 mM formic acid (25:65:10, [v/v]) and absorbance was monitored at 380 nm. The identity of 3E-PΦB was confirmed by co-injection studies. Peak *a* was collected from two identical injections of which one is shown. **B.** Absorbance spectra of peak *a* and BV IXα in acetonitrile-0.1% TFA (60:40, [v/ v]). **C.** Phytochrome difference spectrum (Pfr-Pr) for apophytochrome assembled with peak *a* (+peak *a*). Phytochrome apoprotein was partially purified from *pcd1* tissue (10 g fresh weight) and peak *a* added to the clarified extract to give a final bilin concentration of 0.4 μM (calculated using the absorption coefficient of 3Z-PΦB at 382 nm). Spectra were recorded after incubation of extracts on ice for 30 min and have been mathematically smoothed. The control extract (- peak *a*) was obtained from 2.5 g fresh weight.

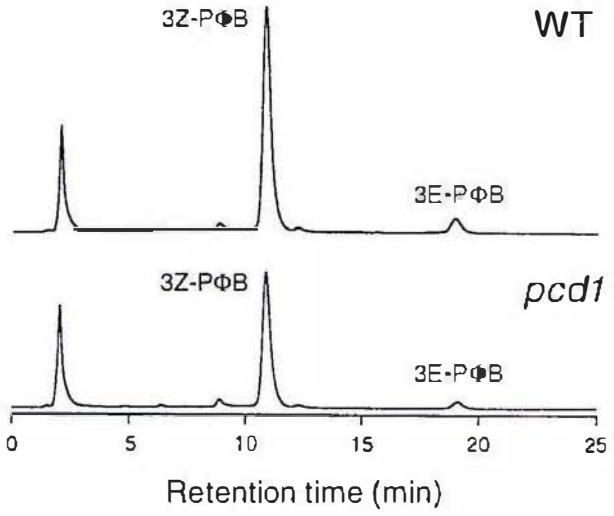

Figure 5.9. HPLC analysis of PΦB synthesis in WT and *pcd1* plastids. Plastids from etiolated WT cv. Solara and *pcd1* seedlings were incubated with 8 μM BV IXα. Incubations were for 3 hr at 28°C and also included an NADPH regenerating system. The final protein concentrations were 1.17 mg/ml for wild-type and 1.16 mg/ml for *pcd1*. The products were analyzed by reverse phase HPLC using a solvent system of ethanol-acetone-water-acetic acid (48:34:17:1, [v/v]) and absorbance was monitored at 380 nm. The identities of 3Z- and 3E-PΦB were confirmed by coinjection studies. Experiment performed by M. Terry.

6. Mutants Deficient in the Phytochrome Chromophore: The *pcd2* Locus

6.1. Introduction

In higher plants, the phytochrome chromophore phytochromobilin (PΦB) is synthesised in the plastid from 5-aminolevulinic acid (ALA, Elich and Lagarias 1987, Terry and Lagarias 1991, Terry et al. 1993b). The steps prior to the formation of protoporphyrin IX are common to both chlorophyll (Chl) and heme synthesis (**Figure 1.5**) and the first committed step in the synthesis of PΦB is the conversion of heme to biliverdin (BV) IXα. This was conclusively demonstrated by the isolation of *pcd1*, a PΦB -deficient pea mutant lacking this activity, as described in the preceding chapter. The subsequent synthesis of 3Z-PΦB from BV IXα has been demonstrated in isolated etioplasts (Terry and Lagarias 1991, Terry et al. 1995), and has recently been shown to be absent in the *aurea* (*au*) mutant of tomato (Terry and Kendrick 1996). The final step in synthesis of the phytochrome chromophore is thought to be the isomerisation of 3Z-PΦB to 3E-PΦB (Terry et al. 1995), but an enzyme activity corresponding to this step has yet to be confirmed.

A feature characteristic of the *pcd1* mutant and all other phytochrome chromophore-deficient mutants described to date is a reduction in sensitivity to both R and FR (**Figure 5.2**, Koornneef et al. 1980, 1985, Parks and Quail 1991, Terry and Kendrick 1996). This reflects reduced activity of both phyA and phyB, since these phytochromes respectively predominate in the sensing of FR and R light (Somers et al. 1991, Parks and Quail 1993, van Tuinen et al. 1995a, 1995b, **Chapters 3, 4**). In screening of mutant pea seedlings (see **Chapter 4**), several mutants showing reduced response to both R and FR were identified, and examined for possible impairment of chromophore synthesis. This chapter describes the isolation and characterisation of *pcd2*, a second chromophore-deficient mutant of pea, which is unable to convert BV IXα to 3Z-PΦB.

6.2. Results

6.2.1. Isolation and Inheritance of the *pcd2* Mutant

As described in Chapter 4, M_2 populations of EMS-mutagenised pea cv. Torsdag were screened under continuous FR to identify mutants defective in FR perception. Mutant AF130 was selected as one among several mutants which showed a dramatic reduction in FR-induced de-etiolation (**Figure 6.1A**), and was subseqently found to also have reduced response to R. AF130 was therefore considered to be a candidate for a mutation causing deficiency in the phytochrome chromophore. Unlike the previously isolated chromophore-deficient *pcd1* mutant, which was strongly chlorotic when grown under cool white fluorescent WL (see **Figure 5.2**), the AF130 mutant is only slightly paler than WT under these conditions (**Figure 6.1B**). Since both the *pcd1* and AF130 mutant phenotypes are strongly expressed in R, allelism tests between *pcd1* and AF130 were conducted under R. **Figure 6.2** shows that F_1 plants of a cross between AF130 and a *pcd1 Le* isolate had internodes similar in length to WT and shorter than either parent, indicating that the AF130 phenotype did not result from mutation at the *PCD1* locus. In F_2 populations of a cross of AF130 to its progenitor line Torsdag (**Figure 6.2**), clear segregation was observed under R (**Figure 6.2**) yielding an overall segregation of 106 WT: 28 AF130-type elongated plants, ($\chi^2_{(3:1)}$ = 1.20, P>0.25). This indicates that the AF130 phenotype is inherited in a manner consistent with a monogenic recessive mutation. The mutant was designated *pcd2*, for *p*hytochrome *c*hromophore *d*eficient 2.

6.2.2. Photomorphogenesis and Phytochrome Content of *pcd2* Seedlings

Phytochrome A is the predominant phytochrome mediating de-etiolation responses to FR in pea seedlings (Chapter 4), while both phyA and phyB have a role in de-etiolation under R (Chapters 3 and 4). Chromophore deficiency results in reduced response to both R and FR (Chapter 5), in keeping with a reduction in activity of both phyA and phyB. **Figure 6.3** shows that *pcd2* mutant plants are essentially blind to FR and show a strongly reduced response to R, while retaining an essentially normal response to blue light (B). The similarity in spectral sensitivity of *pcd2* to *pcd1* suggested that the *pcd2* phenotype might also result from impairment of phytochrome chromophore synthesis. The phytochrome content of *pcd2* mutant plants was therefore examined. **Figure 6.4A** shows representative difference spectra for *in vivo* phytochrome phototransformation in standard samples of etiolated WT and *pcd2* epicotyl tissue. Although samples from WT cv. Torsdag plants routinely contained phytochrome giving a signal of ~25 units (one unit is 10^{-3} $\Delta\Delta A_{660\text{-}730nm}$), the signal in *pcd2* samples was below the detection limit of the

spectrophotometer, which was ~0.3 units. Five-day-old etiolated *pcd2* seedlings therefore contain <1.5% of the spectrally active phytochrome detectable in the WT. Since the majority of spectrophotometrically detectable phytochrome in etiolated WT tissue is phyA (Parks and Quail 1993; van Tuinen et al. 1995a), this severe deficiency in spectrally active phytochrome in *pcd2* seedlings could conceivably result from a specific deficiency in the phyA apoprotein. We therefore also examined the phytochrome apoprotein content of the *pcd2* mutant. However, immunoblot analysis of crude protein extracts showed that etiolated *pcd2* seedlings have essentially normal levels of the phyA apoprotein (**Figure 6.4B**). The depletion of phyA apoprotein levels following exposure to R provides an additional assay for phytochrome spectral activity since phyA is synthesised in the P_r form and depletion is dependent on photoconversion to P_{fr} (Quail et al. 1973). **Figure 6.4B** shows that phyA apoprotein in *pcd2* seedlings was only depleted to a slight extent during a 4 h R treatment in contrast to the strong depletion seen in WT seedlings. This result provides further evidence that the majority of phyA in *pcd2* mutant seedlings is unable to undergo photoconversion from P_r to P_{fr}, consistent with a deficiency in chromophore synthesis.

6.2.3. Phytochromobilin Synthesis in *pcd2* Plastids

The first committed step in synthesis of the phytochrome chromophore, 3*E*-PΦB, is the conversion of heme to BV IXα (**Figure 1.5**; Terry et al. 1993; **Chapter 5**). As this step is specifically impaired in the *pcd1* mutant (**Chapter 5**), it was therefore considered that the *pcd2* mutation might affect the subsequent conversion of BV IXα to 3*Z*-PΦB (Figure 1). This possibility was examined using a previously described HPLC-based assay system for PΦB synthesis in plastids isolated from etiolated seedlings (Terry et al. 1995; **Chapter 5**). **Figure 6.6** shows that WT plastids convert BV IXα to two products (**trace d**), identified by coinjection and absorbtion spectroscopy as 3*Z*-PΦB and 3*E*-PΦB (data not shown; **Chapter 5**). In contrast, incubation of *pcd2* plastids with BV IXα yielded only a single peak (**trace e**), which was distinct from co-injected 3*Z*-PΦB (data not shown), and corresponded to unmetabolised BV IXα. These data clearly demonstrate that the *pcd2* mutant is unable to convert BV IXα to 3*Z*- or 3*E*-PΦB. To confirm the viability of the plastids used in this experiment, the metabolic fate of heme in the same plastid preparations was also examined (**Figure 6.7**). Whereas WT plastids formed both 3*Z*-PΦB and 3*E*-PΦB from heme (**Figure 6.7, trace b; Chapter 5**), *pcd2* plastids formed only a single product (**trace c**) which co-eluted with BV IXα (**trace d**) but not with 3*Z*-PΦB (**trace e**). The *pcd2* mutant plastids are therefore able to accomplish the conversion of heme to BV IXα, but not the further conversion of BV IXα to 3*Z*- and 3*E*-PΦB.

6.2.4. Comparison of *pcd1*, *pcd2* and *pcd1 pcd2* Double Mutant Phenotypes

Given that *pcd1* and *pcd2* both impose a severe block to PΦB synthesis, the two mutants might be expected to show a very similar phenotype. Although both mutants show similar de-etiolation responses under monochromatic light (**Figure 5.2, 6.3**), a notable difference was apparent between *pcd1* and *pcd2* plants grown in continuous WL. Whereas *pcd1* plants have strikingly pale, yellow-green foliage under these conditions (**Figure 5.1**), the *pcd2* mutant was only slightly paler than WT (**Figure 6.1B**). To compare the effects of the *pcd1* and *pcd2* mutants on a similar genetic background, we backcrossed the *pcd1* mutant twice from the cv. Solara background into the cv. Torsdag background and used a random selection of F_4 seed from the second back-cross for comparison with *pcd2*. Comparable *pcd1 pcd2* double mutant seed was produced by a similar strategy and was also included in these experiments. **Figures 6.8** and **6.9** show that the *pcd2* mutation has only a slight effect on leaflet Chl content in seedlings grown under continuous WL, whereas in *pcd1* and the *pcd1 pcd2* double mutant, total Chl content is reduced and the Chl *a*/*b* ratio is substantially increased. The effects of the *pcd2* mutation are more reminiscent of those seen in phyB-deficient *lv* mutants, which show a relatively minor reduction in Chl content and no alteration to the Chl *a*/*b* ratio (Nagatani et al. 1990). Interestingly, *pcd2* has a much stronger effect on Chl content in the stem than in the leaf, conferring reduction in total Chl equivalent to that seen in *pcd1*, whether expressed in terms of fresh weight or per internode (**Figure 6.9**). However, in the stem, as in the leaflets, *pcd2* does not alter the Chl *a*/*b* ratio, whereas *pcd1* confers a significant increase.

Phytochrome has been shown to have an important influence on both Chl synthesis (Kasemir 1983) and the expression of genes coding for Chl *a*/*b* -binding proteins (Batschauer et al. 1994). Therefore, one possible explanation for the difference in Chl content of *pcd1* and *pcd2* is that the *pcd1* mutation has a more severe effect on PΦB synthesis and hence on the level of active, photoreversible phytochrome. Under our assay conditions, neither mutant had detectable holophytochrome (**Figures 6.4A, 5.4A**) or PΦB synthesis from heme (**Figures 6.7, 5.7**). However, both *pcd1* and *pcd2* clearly retain some degree of responsiveness to R (**Figures 6.3, 5.2**). The response to R may thus be a more sensitive measure of active phytochrome in the mutants than the spectrophotometric assay, and we therefore compared the de-etiolation responses of *pcd1*, *pcd2* and the *pcd1 pcd2* double mutant under continuous R, B and WL (**Figure 6.10**). The fact that the *pcd1 pcd2* double mutant is more etiolated under R than either single mutant confirms that both single mutants are somewhat leaky. However, **Figure 6.10** shows that in a similar genetic background, the *pcd1* and *pcd2* mutations confer a similar reduction in de-etiolation responses to R. This suggests that the mutations impose an equivalently severe depletion

of phytochrome, and it therefore appears unlikely that the difference in Chl content is due to differing effects of *pcd1* and *pcd2* on phytochrome content.

Both *pcd1* and *pcd2* showed a much smaller reduction in sensitivity to B than to R (**Figure 6.10**). This suggests that phytochrome has only a relatively minor role in de-etiolation under B, and influences leaf development more than stem elongation. In addition, the *pcd1* and *pcd2* mutations fail to show an additive effect on stem elongation under B, with the *pcd1 pcd2* double mutant slightly more responsive than either single mutant. This somewhat unexpected result is also seen under WL, where stem elongation in double mutant seedlings is inhibited to a greater extent than in *pcd1* or *pcd2* alone (**Figures 6.8, 6.10**). In contrast to the situation for the control of stem elongation, *pcd1* and *pcd2* showed quite a strong additive effect on leaf expansion under WL (**Figure 6.10**). This effect was also seen under B, but was less pronounced.

6.3. Discussion

Phytochromobilin Synthesis in the *pcd2* Mutant

Pea mutants have now been identified for the first two committed steps in PΦB biosynthesis. The *pcd1* mutant is severely deficient in spectrally active phytochrome, due to an inability to synthesise BV IXα from heme (**Chapter 5**). The subsequent reduction of BV IXα to 3Z-PΦB is accomplished by the plastid-localised enzyme PΦB synthase (Terry and Lagarias 1991; Terry et al. 1995). Plastid preparations from *pcd2* tissue are clearly unable to synthesise 3Z-PΦB from heme (**Figure 6.7**) or from BV IXα (**Figure 6.6**), but support the conversion of heme to BV IXα (**Figure 6.7**). The severe deficiency in spectrally active phytochrome in *pcd2* therefore appears to result from a specific impairment of PΦB synthase activity. PΦB synthase has yet to be purified but it is expected to be similar in nature to the ferredoxin-dependent enzyme required for the analogous reduction of 15,16-dihydroBV to 3Z-phycoerythrobilin in the red alga *Cyanidium caldarium* (Beale and Cornejo 1991). Although both 3Z-PΦB and 3*E*-PΦB are detected as products of BV IXα metabolism (Terry et al. 1995; **Figure 6.7**), the 3Z- isomer is formed more rapidly, implicating the presence of an isomerase catalysing the conversion of 3Z- to 3*E*-PΦB. It would be interesting to know if *pcd2* plastids retained a PΦB isomerase activity.

Phenotypes of the *pcd1*, *pcd2* and *pcd1 pcd2* Double Mutants

With respect to de-etiolation responses under monochromatic light, the *pcd2* mutant is very similar to the *pcd1* mutant. Both mutants are essentially unresponsive to FR, show a substantial reduction in response to R, and show only a small reduction in response to B. These results are consistent with a deficiency in both phyA and phyB at the seedling stage. The most striking difference between *pcd1* and *pcd2* is in leaflet Chl content. The more chlorotic phenotype of *pcd1* plants is unlikely to be due to a more severe phytochrome deficiency, since by all criteria measured the effect of *pcd2* on phytochrome levels and responses is at least as severe as *pcd1* (**Figures 6.3, 6.4, 6.7, 6.10, Chapter 5**). The *pcd2* mutant clearly shows that phytochrome deficiency sufficiently severe to cause a dramatic reduction in de-etiolation responses does not in itself confer a large reduction in Chl content. The severe chlorosis seen in *pcd1* plants must therefore have an alternative explanation relating to the specific impairment of the heme to BV IXα conversion. Heme is known to be an inhibitor of ALA synthesis (Beale and Weinstein 1991), and one likely possibility therefore is that *pcd1* plants accumulate heme, leading to a reduction in ALA synthesis and consequently to reduced synthesis of Chl. In addition, it is possible that additional toxic effects of accumulated heme or heme precursors may also contribute to the Chl deficiency of *pcd1*, although the plants show no other obvious symptoms of toxicity, and *pcd2* plants might also be expected to show similar effects.

Although no photoreversible phytochrome was detected in etiolated *pcd1* or *pcd2* seedlings, the retention of sensitivity to R indicates the presence of active phytochrome and hence that the blocks to PΦB synthesis imposed by both mutations are incomplete. In addition, the effect of *pcd2* on stem elongation under WL diminishes with age (**Figure 6.5**), in a manner similar to *pcd1* (**Figure 5.3**) and unlike the phyB-deficient *lv* mutants, in which the elongated phenotype persists with age. Leakiness of both *pcd1* and *pcd2* is confirmed by the fact that the *pcd1 pcd2* double mutant is even less responsive to R than either single mutant (**Figure 6.10**). The responses of *pcd1*, *pcd2* and the *pcd1 pcd2* double mutant to continuous monochromatic light also appear to be in keeping with the functions of phyA and phyB deduced from similar experiments with the *fun1-1 lv-5* double mutant (**Figure 4.19**). The increased response to B and WL in the *pcd1 pcd2* double mutant relative to either single mutant is likely to reflect the strong deficiency of functional phyA, given the apparent stimulatory effect of phyA on stem elongation under B (**Chapter 4**).

Comparison of *pcd1* and *pcd2* with Other Chromophore-Deficient Mutants

In addition to *pcd1* and *pcd2*, mutants deficient in the phytochrome chromophore have been identified in both tomato (*yg*-2 and *au*; Koornneef et al. 1985) and *Arabidopsis* (*hy1*, *hy2* and *hy6*; Koornneef et al. 1980; Chory et al. 1989a). Like *pcd1* and *pcd2*, the tomato and *Arabidopsis* mutants also show defects in de-etiolation under both R and FR (Koornneef et al. 1980, 1985), consistent with the reduction in the activity of both phyA and phyB expected to result from chromophore deficiency. The *yg*-2 mutation of tomato has recently been shown to impair the conversion of heme to BV IXα and is thus equivalent to *pcd1*, while the *au* mutant is blocked between BV IXα and 3Z-PΦB (Terry and Kendrick 1996) and is therefore analogous to *pcd2*. The exact sites of action of the *Arabidopsis* mutations have yet to be resolved. However, the *hy1* mutant is fully rescued by BV, whereas rescue of the *hy2* mutant is incomplete (Parks and Quail 1991), which suggests that *hy1* and *hy2* may correspond to *pcd1* and *pcd2*, respectively. Putative chromophore-deficient mutants identified on the basis of partial rescue by BV have also been reported in *Nicotiana plumbaginifolia* (Kraepiel et al. 1994) and the moss *Ceratodon purpureus* (Lamparter et al. 1996).

The main inconsistency between the pea and tomato mutants is the chlorotic phenotype. Whereas *pcd2* shows only a slight reduction in total Chl and has no effect on the Chl *a*/*b* ratio (**Figure 6.9**), the analogous *au* mutant displays the yellow-green chlorotic phenotype of *pcd1* and *yg*-2 (Koornneef et al. 1985; van Tuinen et. al. 1996a; **Figure 5.1**). *De novo* synthesis of protochlorophyllide is reduced in etiolated *au* and *yg*-2 seedlings (M.J.Terry, pers. comm.), suggesting that a light-independent reduction of Chl synthesis may make a substantial contribution to the chlorotic phenotype. This is consistent with effects expected from feedback regulation of ALA synthesis by heme. The dramatic difference between the *au* and *pcd2* mutants might therefore relate to relatively subtle differences in heme accumulation and/or differences in sensitivity to down-regulation of Chl synthesis by heme. Although both the *hy1* and *hy2* mutants are also variously reported to be light- or yellow-green (Koornneef et al. 1980; Chory et al. 1989a), a comparison of *hy1* and *hy2* alleles null for spectrally detectable phytochrome showed *hy2* to have a less severe effect on Chl content than *hy1* (Chory 1992). Thus it is possible that the severe, yellow-green phenotype is more directly associated with an impairment of the heme to BV IXα conversion. Further work, and the identification of true null alleles will obviously be required to fully resolve this issue. In any event, the *pcd1* and *pcd2* mutants characterised here, together with similar mutants of tomato and *Arabidopsis*, constitute a promising resource for a combined physiological, biochemical and molecular approach to investigation of the regulation of tetrapyrrole biosynthesis.

6.4. Methods

6.4.1. Plant Material and Growth Conditions

The AF130 mutant was identified in an M_2 population grown under 8 μmol m^{-2} sec^{-1} FR (see **Chapter 4**). The mutant line was bred by single plant selection for two generations before backcrossing to cv. Torsdag. All *pcd2* material in this study was derived from bulked *pcd2* segregates in the F_3 and F_4 generations of this cross. The *pcd1* mutant was originally isolated in cv. Solara (genotype *le i af*; see **Chapter 5**). The *pcd1* material used in allelism testing (**Figure 6.2**) was bulked from F_2 segregates after two successive backcrosses from cv. Solara into cv. Torsdag (genotype *LE I AF*). Mutant *pcd1* plants with genotype *LE I AF* obtained after only one such back-cross were used in a cross to the original AF130 line to obtain the *pcd1 pcd2* double mutant. Double mutant seedlings were readily identified as pale segregates from *pcd2* families in the F_3 of this cross. The WT, *pcd1*, *pcd2*, and *pcd1 pcd2* plants used for the monochromatic light experiments (**Figures 6.8, 6.10**) and for Chl measurement (**Figure 6.9**) were a random selection of F_4 segregates from this same cross.

After initial screening under R or WL, plants for all genetic experiments (**Figure 6.2**) were grown to maturity in a heated glasshouse under a natural photoperiod extended to 18 h with light from a mixed fluorescent/incandescent source. Plants for biochemical experiments (**Figures 6.4, 6.6, 6.7**) were sown in water-saturated vermiculite and grown in complete darkness at 25°C. The standard RIKEN R source was used to deplete phytochrome in the immunoblotting experiment (**Figure 6.4B**). For all other experiments, plants were grown in growth cabinets at 20°C, in standard Hobart pea potting mix using standard Hobart light sources. Standard green safety light was used for manipulation of etiolated plants and during all biochemical experiments.

6.4.2. Other Methods

Methods for reagent preparation, PΦB synthesis assays, Chl quantification, immunoblotting and spectrophotometric assay for phytochrome were followed as described in previous chapters.

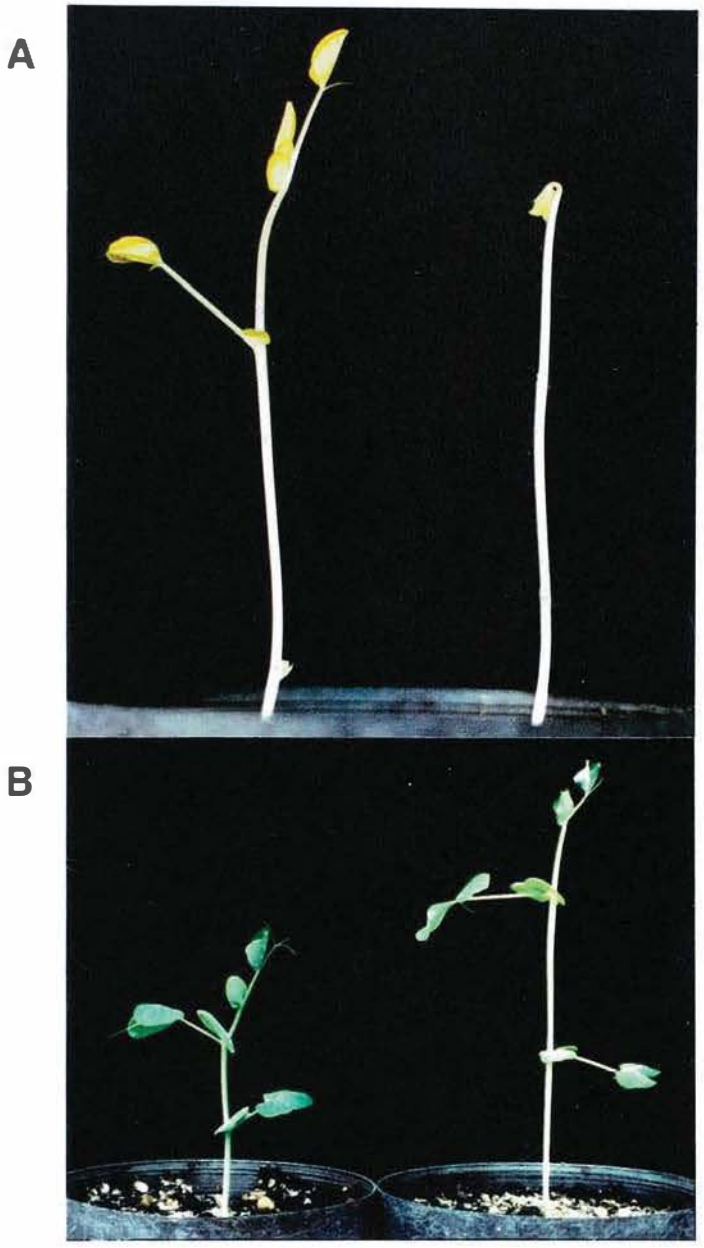

Figure 6.1. Representative WT cv. Torsdag (left) and AF130 mutant seedlings. **A.** Seedlings grown in FR (8 μmol m^{-2} sec^{-1}) for 7 days. **B.** Seedlings grown in WFL (150 μmol m^{-2} sec^{-1}) for 10 days.

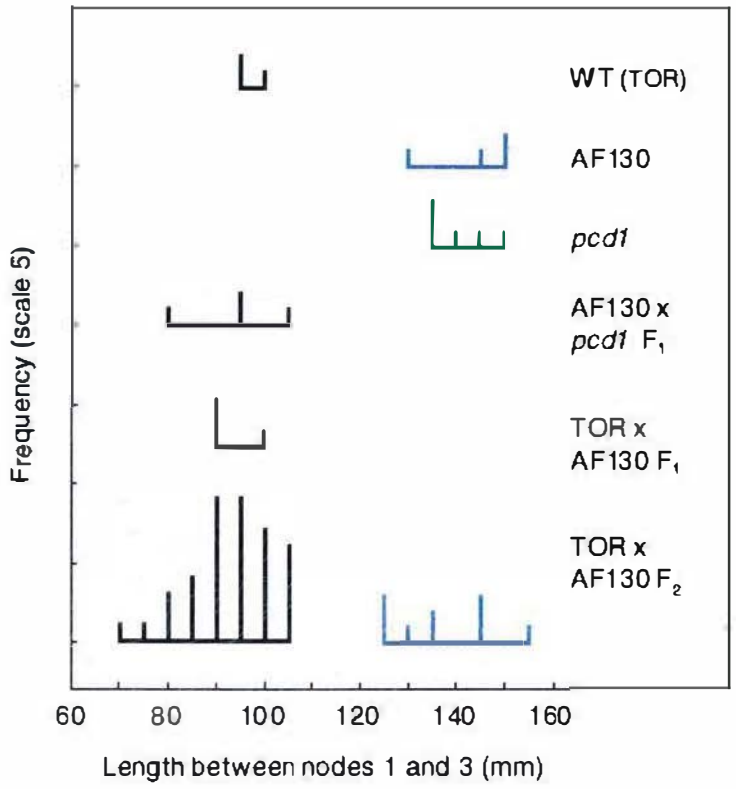

Figure 6.2. Genetic analysis of the AF130 phenotype. Frequency distributions for stem length are shown for WT cv. Torsdag (TOR), AF130 and *pcd1* mutant seedlings, and for progeny of crosses AF130 x *pcd1* and TOR x AF130. All plants were grown in R (20 μmol m^{-2} sec^{-1}). Black, blue and green bars represent seedlings with WT, AF130 and *pcd1* phenotypes, respectively.

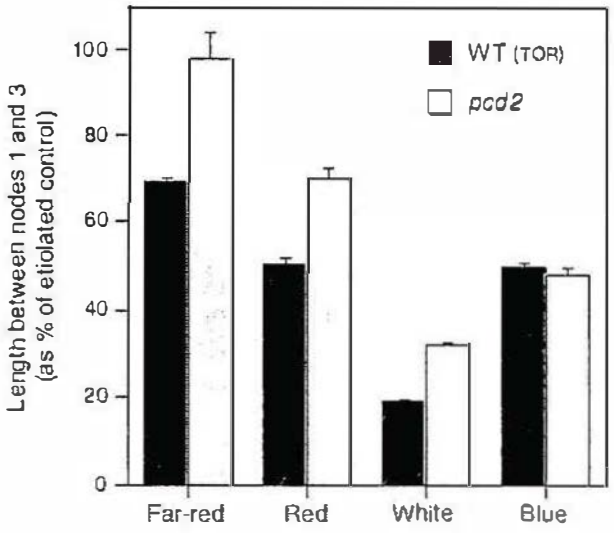

Figure 6.3. Stem elongation of the *pcd2* mutant grown under monochromatic light. WT cv. Torsdag (TOR) and *pcd2* mutant seedlings were grown in darkness or under FR (8 μmol m^{-2} sec^{-1}), R (20 μmol m^{-2} sec^{-1}), WFL (150 μmol m^{-2} sec^{-1}) or B (10 μmol m^{-2} sec^{-1}) at 20°C. Error bars represent SE, n=8-12.

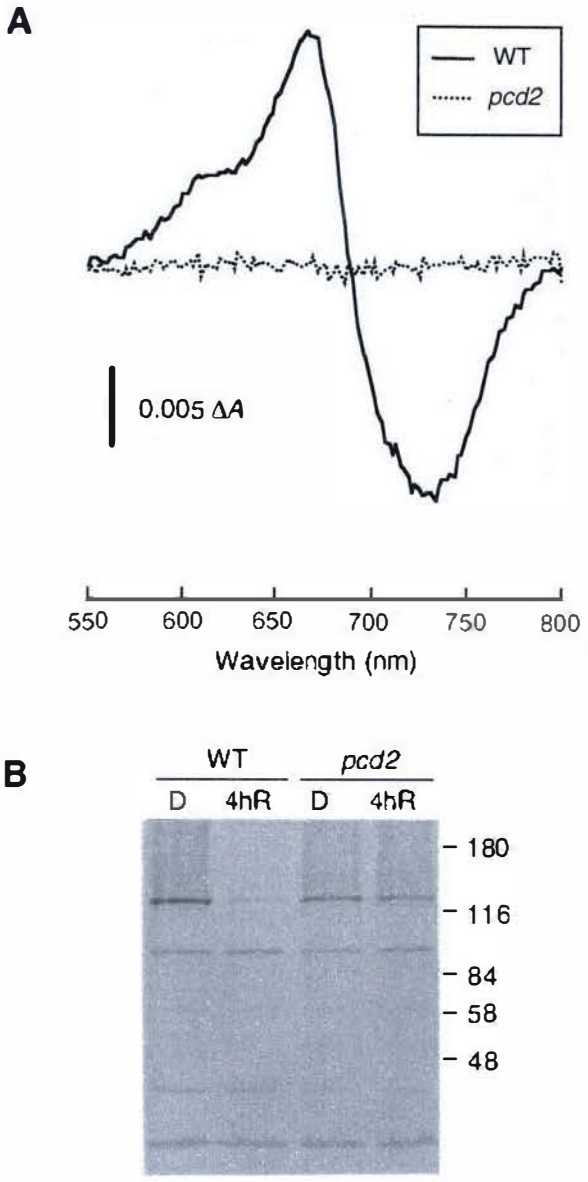

Figure 6.4. Phytochrome content of *pcd2* seedlings. **A.** Difference spectrum for *in vivo* phytochrome phototransformation (P_{fr}-P_r) in etiolated WT cv. Torsdag and *pcd2* mutant seedlings. **B.** Immunodetection of PHYA apoprotein following SDS-PAGE and western blotting of crude protein extracts from WT and *pcd2* seedlings grown in complete darkness (D) or given a 4 h R treatment (17 μmol m^{-2} sec^{-1}) immediately before harvest. Lanes were loaded on an equivalent fresh weight basis. The positions and molecular masses (kD) of prestained markers (Sigma) are indicated.

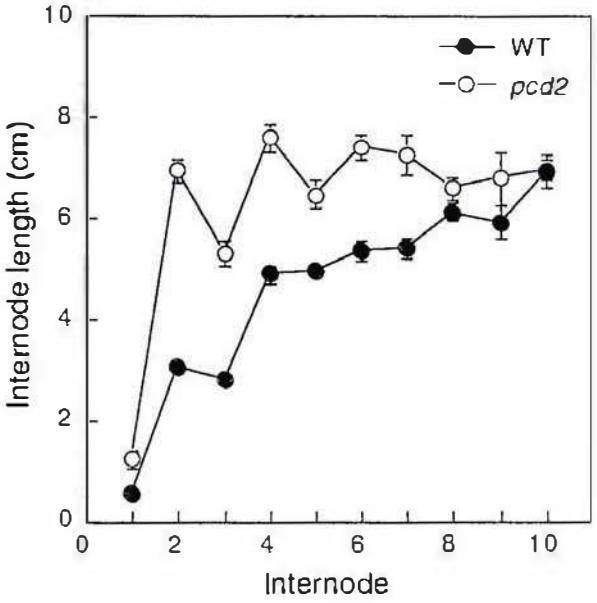

Figure 6.5. Internode lengths of WT cv. Torsdag and *pcd2* mutant plants grown under continuous WFL (150 μmol m^{-2} sec^{-1}). Error bars represent SE, n=6.

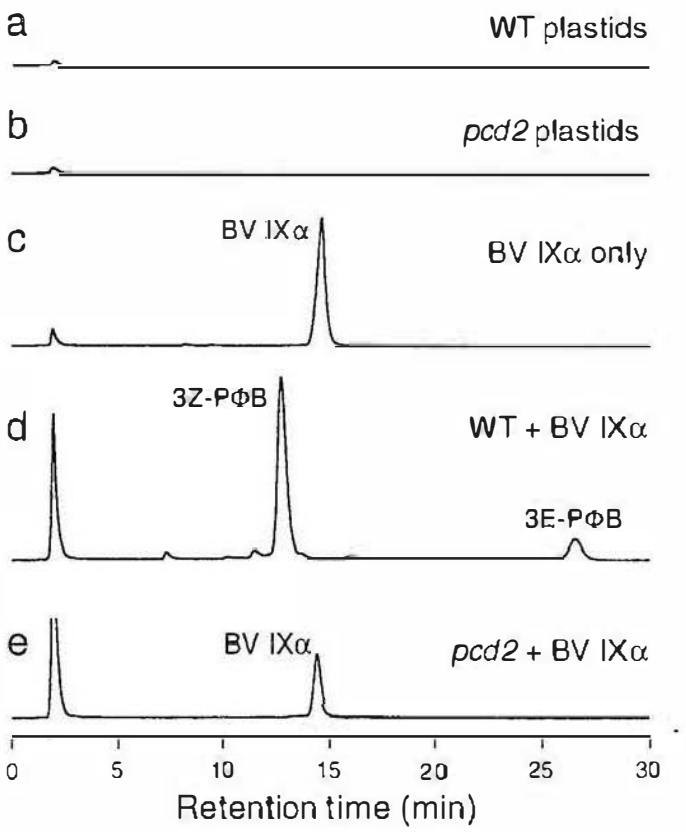

Figure 6.6. HPLC analysis of PΦB synthesis from BV IXα in *pcd2* . Plastids from etiolated WT cv. Torsdag and *pcd2* seedlings were incubated with 8 μM BV IXα and a NADPH regenerating system for 3 h at 28°C. The products were analyzed by reverse-phase HPLC using a solvent system of ethanol-acetone-100mM formic acid (25:65:10, [v/v]) and absorbance was monitored at 380 nm. Trace a, WT plastids (0.78 μg ml^{-1}) in the absence of BV IXα; trace b, *pcd2* plastids (0.80 μg ml^{-1}) in the absence of BV IXα; trace c, BV IXα in the absence of plastids; trace d, WT plastids (0.78 μg ml^{-1}) incubated with BV IXα; trace e, *pcd2* plastids (0.80 μg ml^{-1}) incubated with BV IXα. For trace c the injection volume was half that used for the other samples and an the absorbance scale is twice that of the other traces. The absence of 3Z-PΦB in the *pcd2* + BV IXα sample (trace e) was confirmed by co-injection. Experiment performed by M. Terry.

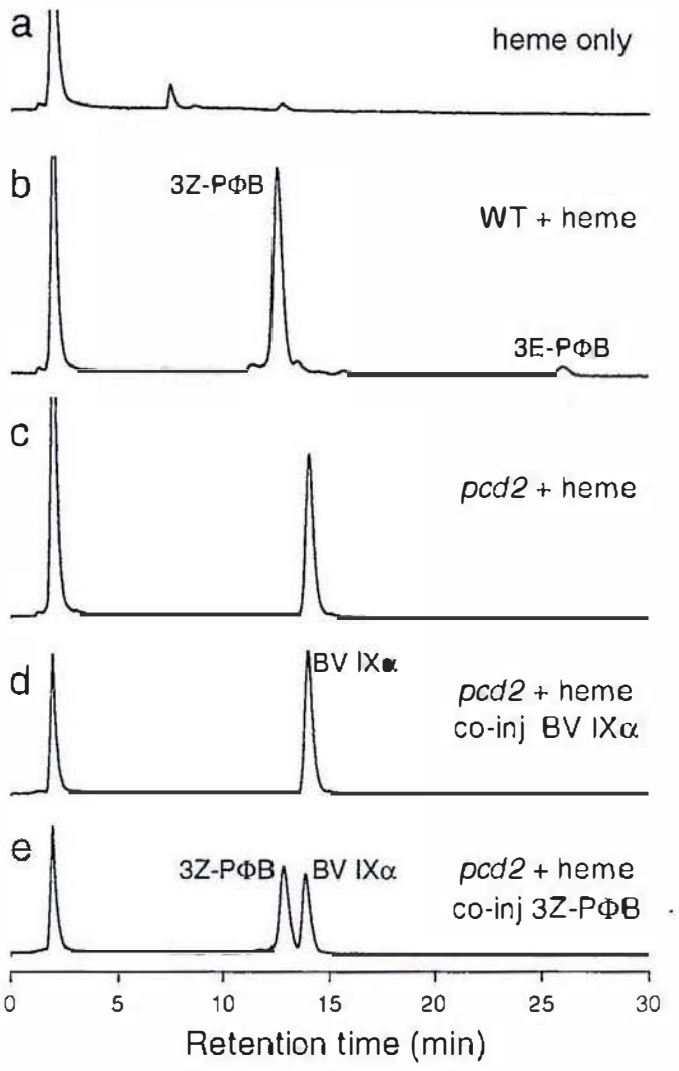

Figure 6.7. HPLC analysis of PΦB synthesis from heme in *pcd2*. Plastids from etiolated seedlings of WT cv. Torsdag and *pcd2* were incubated with 10 μM heme and a NADPH regenerating system for 3 h at 28°C. The products were analyzed by reverse-phase HPLC using a solvent system of ethanol-acetone-100mM formic acid (25:65:10, [v/v]) and absorbance was monitored at 380 nm. Trace a, heme in the absence of plastids; trace b, WT plastids (0.78 μg ml^{-1}) incubated with heme; trace c, *pcd2* plastids (0.80 μg ml^{-1}) incubated with heme; trace d, *pcd2* plastids incubated with heme (2/5 the volume used in trace c) co-injected with 15 pmol BV IXα; trace e *pcd2* plastids incubated with heme (2/5 the volume used in trace c) co-injected with 50 pmol 3Z-PΦB. Experiment performed by M. Terry.

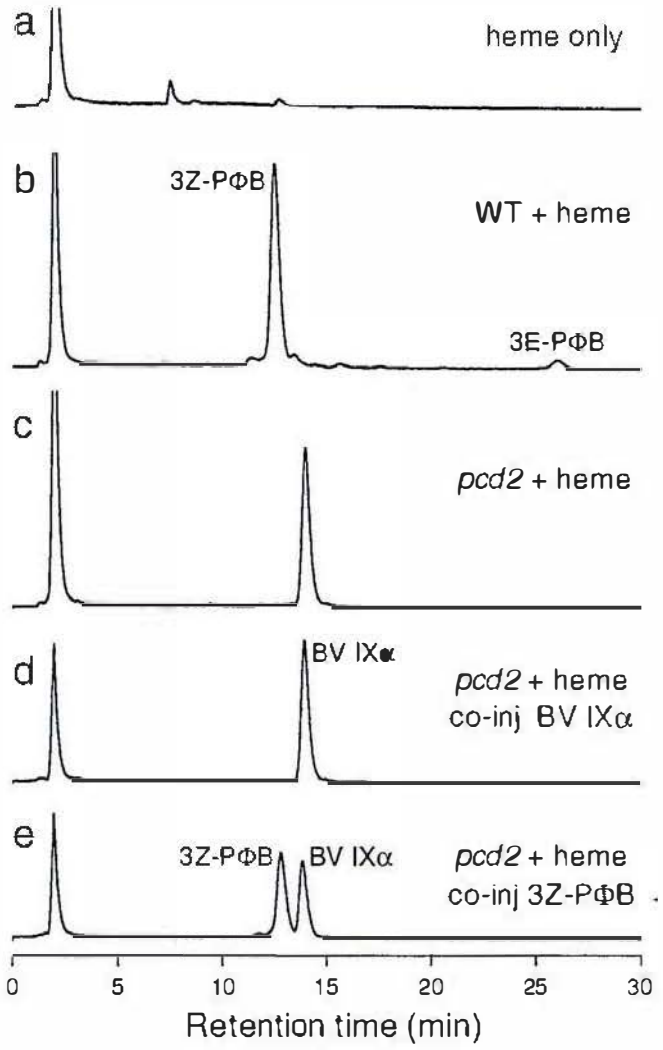

Figure 6.7. HPLC analysis of PΦB synthesis from heme in *pcd2*. Plastids from etiolated seedlings of WT cv. Torsdag and *pcd2* were incubated with 10 μMheme and a NADPH regenerating system for 3 h at 28°C. The products were analyzed by reverse-phase HPLC using a solvent system of ethanol-acetone-100mM formic acid (25:65:10, [v/v]) and absorbance was monitored at 380 nm. Trace a, heme in the absence of plastids; trace b, WT plastids (0.78 μg ml^{-1}) incubated with heme; trace c, *pcd2* plastids (0.80 μg ml^{-1}) incubated with heme; trace d, *pcd2* plastids incubated with heme (2/5 the volume used in trace c) co-injected with 15 pmol BV IXα; trace e *pcd2* plastids incubated with heme (2/5 the volume used in trace c) co-injected with 50 pmol 3Z-PΦB. Experiment performed by M. Terry.

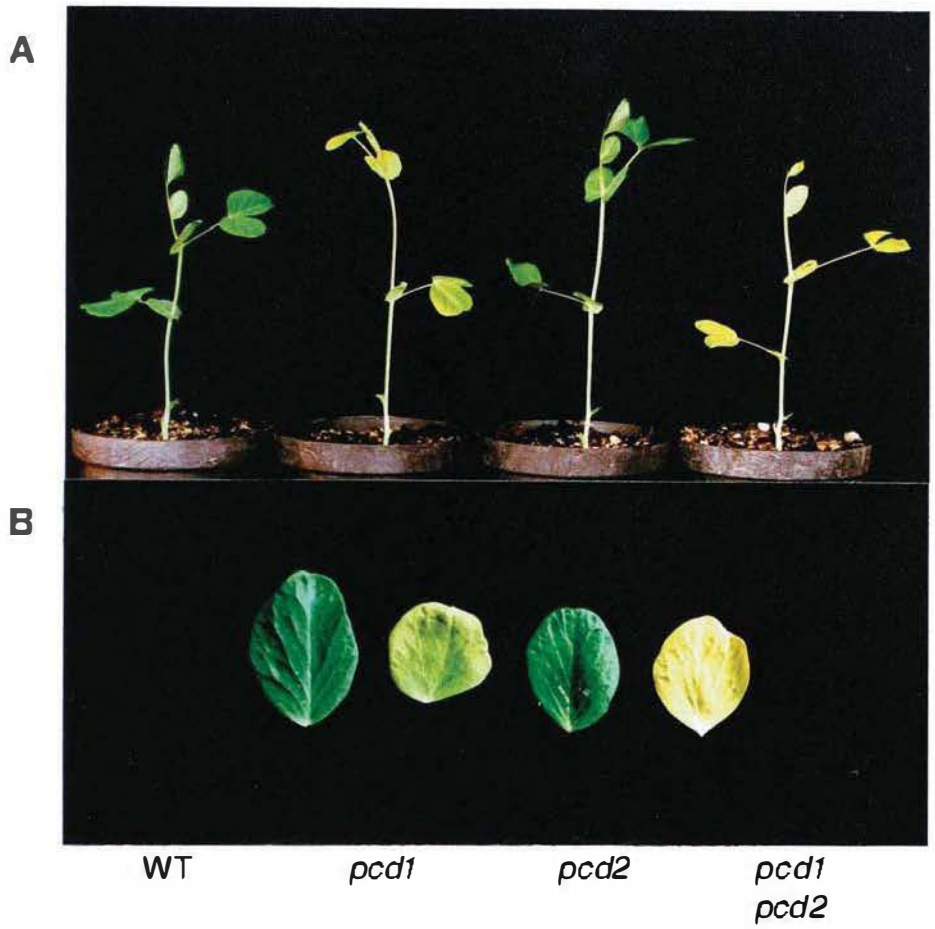

Figure 6.8. Phenotypes of WT cv. Torsdag, *pcd1*, *pcd2* and *pcd1 pcd2* double mutant seedlings grown under continuous WFL (150 μmol m^{-2} sec^{-1}). **A.** Seedlings at 10 days after sowing. **B.** Representative leaflet from leaf 4.

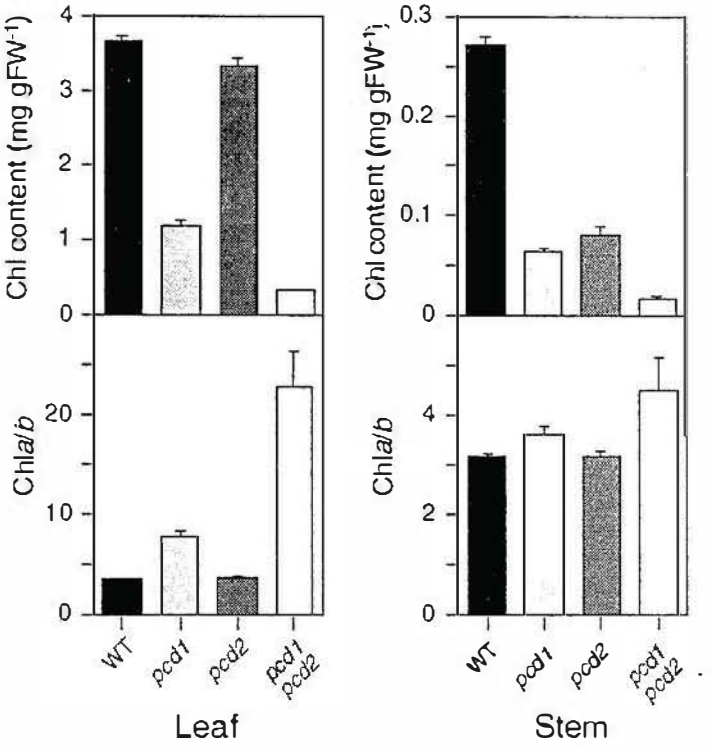

Figure 6.9. Chlorophyll content of *pcd1*, *pcd2* and the *pcd1 pcd2* double mutant. WT cv. Torsdag, *pcd1*, *pcd2* and *pcd1 pcd2* double mutant seedlings were grown under continuous WL (150 μmol m^{-2} sec^{-1}). Total Chl content (mg gFW^{-1}) and Chl *a* /*b* ratio were determined in samples of leaflet (leaf 4) and stem tissue (internode 3). The total Chl content of internode 3 was 22.9±1.3 μg (WT), 13.1±1.1 μg (*pcd1*), 15.0±1.0 μg (*pcd2*) and 3.1±0.3 μg(*pcd1 pcd2*). Error bars represent SE, n=6-8.

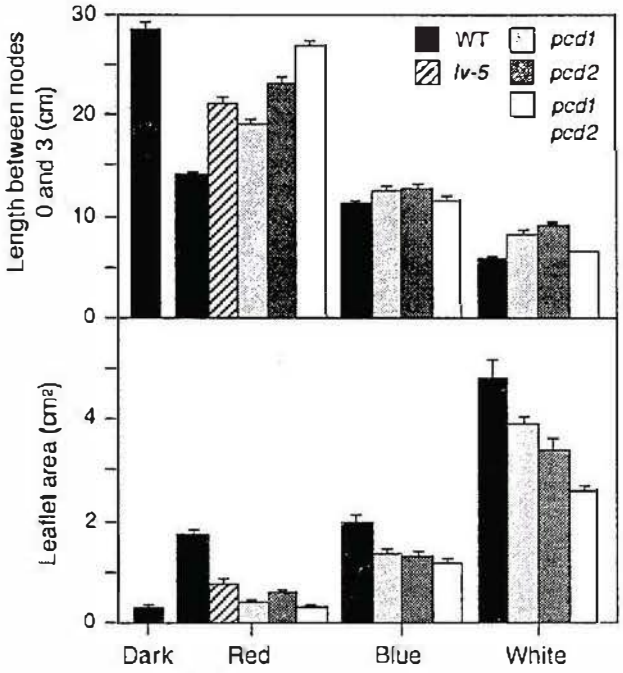

Figure 6.10. Comparison of de-etiolation responses in *pcd1*, *pcd2* and the *pcd1 pcd2* double mutant. WT cv. Torsdag, *pcd1*, *pcd2* and *pcd1 pcd2* double mutant seedlings were grown under continuous R (20 μmol m^{-2} sec^{-1}), B (10 μmol m^{-2} sec^{-1}) or WFL (150 μmol m^{-2} sec^{-1}). **A.** Stem elongation. **B.** Leaflet area, estimated as the product of the length and breadth of the larger leaflet of the first true foliage leaf (node 3). Error bars represent SE, n=8-12.

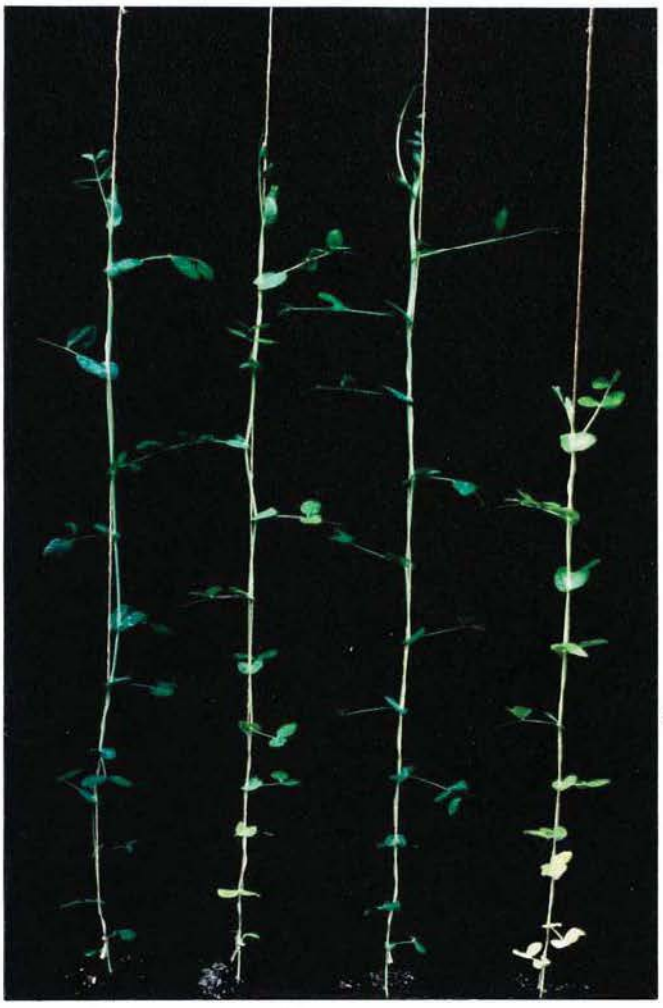

Figure 6.11. Phenotypes of six-week-old WT cv. Torsdag, *pcd1*, *pcd2* and *pcd1 pcd2* double mutant plants grown under standard glasshouse conditions.

7. A Mutant with Exaggerated Light Responses: The AF05 Mutant

7.1. Introduction

Isolation and characterisation of mutants affecting plant responses to light has been an integral part of recent advances in photomorphogenesis research (Quail et al. 1995, Smith 1995, Ahmad and Cashmore 1996). Those isolated to date include mutants deficient in phytochrome (Somers et al. 1991, Parks and Quail 1993, van Tuinen et al. 1995a, 1995b, Chapters 3 and 4) and cryptochrome photoreceptors (Ahmad and Cashmore 1993, Lin et al. 1995a, 1995b), as well as a number of mutants which are insensitive to various wavebands but do not appear to affect photoreceptor function directly. These latter mutants, which include the *hy5*, *fhy1*, *fhy3* and *elf3* mutants of *Arabidopsis* (Koornneef et al. 1980, Zagotta et al. 1992, Whitelam et al. 1993) are currently considered to impair photoreceptor signal transduction in some way. Transgenic overexpression of photoreceptor genes also results in a light-dependent phenotype, but one characterised by exaggerated responses to light. For example, phyB overexpressors are more sensitive to R, while phyA overexpressors are hypersensitive to both R and FR (McCormac et al. 1993). Although a small number of light-hypersensitive mutants have been described, induced mutations conferring elevated photoreceptor gene expression or photoreceptor levels have not been reported. The *hp-1* mutant of tomato is hypersensitive to R and FR (Peters et al. 1989, 1992) but is not linked to any of the known phytochrome genes of tomato (van Tuinen et al. 1996b). Also, the mutant is inherited in a recessive manner, which contrasts the semi-dominant inheritance seen for photoreceptor genes (Koornneef et al. 1980, Whitelam et al. 1993, van Tuinen et al. 1995a, 1995b). These characteristics suggest that *hp-1* is unlikely to affect photoreceptor activity directly, but probably impairs signal transduction in some way (Peters et al. 1992).

In addition, mutants have now been identified which confer a light-independent de-etiolated phenotype. Although such mutants are not, strictly speaking, photomorphogenic, they have been important in demonstrating the existence of genes required for maintenance of the etiolated habit. While many mutants in this broad and heterogenous class are known from *Arabidopsis* (including *cop*, *det* and *fus* mutants; Chory et al. 1989b, Deng et al. 1991, Misera et al. 1994) the only other similar mutant so far identified is the *lip1* mutant of pea (Frances et al. 1992). When grown in the dark, mutants of this class have phenotypes which are to a varying extent reminiscent of light-grown

WT plants, with increased inhibition of stem elongation, increased leaf development, and elevated expression of light-regulated genes. In addition to a de-etiolated phenotype in darkness, *lip1* plants have also been observed to show a more fully de-etiolated phenotype than WT under monochromatic R, B and FR.

Given the existence of so few mutants with exaggerated light responses, and the existence of so few constitutively photomorphogenic mutants other than those in *Arabidopsis*, I was interested to know whether additional mutants in either class could be isolated from pea. Since representatives from both classes (e.g. *hp-1* of tomato and *det1* of *Arabidopsis*) are expressed under both R and FR (Peters et al. 1992, Chory 1992), these conditions seemed appropriate for selection of mutants in both classes, as well as mutants overexpressing phyA or phyB. Therefore, as a routine part of screening for mutants with reduced response to R or FR, mutants more strongly de-etiolated than WT were also selected. This chapter reports on the isolation and characterisation of one such mutant, AF05, which was selected for a dwarf, de-etiolated phenotype in screens under both R and FR.

7.2. Results

7.2.1. Isolation and Inheritance of the AF05 mutant

The AF05 mutant was first selected in a screen under FR, where it was clearly distinguishable from WT plants on the basis of short internodes and an advanced state of leaf development (**Figure 7.1**). This phenotype is very similar to that of the *lip1* mutant grown under the same conditions (**Figure 7.1**). The AF05 mutant was also identified when M_2 plants from the same M_1 family were screened under R, and under these conditions the effects of the mutation on elongation and leaf expansion were equally as dramatic as under FR (see **Figure 7.4B**). R conditions were predominantly used for genetic analysis of the mutant, as seedlings grown under R survived somewhat better after transfer to normal glasshouse conditions than those grown in FR.

When the M_3 progeny of the AF05 mutant were grown under R, three distinct phenotypes were apparent. Approximately half of the population was very similar in phenotype to the original AF05 mutant. Of the remainder, some plants were even shorter and more de-etiolated than the AF05 parent, while others were virtually indistinguishable from WT. The approximate 1 short: 2 intermediate: 1 WT segregation suggested the possibility that the mutation might be dominant, with the intermediate class of M_3 segregates and the original AF05 mutant representing heterozygous plants. This would predict that the

dwarf and WT segregates should breed true and the intermediate class should again segregate all three phenotypes in a 1:2:1 ratio. This prediction was verified by growing various M_4 families under R (**Figure 7.2**). For final confirmation, the F_2 of a cross between a dwarf, putative homozygous dominant mutant plant and cv Torsdag was grown. **Figure 7.3A** shows that as expected the F_2 segregated in accord with a 1:2:1 ratio under both FR (χ^2=1.17, P>0.25) and R (χ^2=1.06, P>0.3). These results establish that the AF05 phenotype results from an incompletely dominant mutation. The degree of dominance for the AF05 mutant allele calculated on the basis of the results in **Figure 7.3A** was 34% under FR and 38% under R. Interestingly, the degree of dominance of the mutant allele appeared to vary with development of the plant. Under FR, the AF05 allele was almost completely dominant in controlling elongation of the first internode, but in subsequent internodes, approached co-dominance with the WT allele (**Figure 7.3B**).

Although the original AF05 mutant was heterozygous, all experiments described below used a homozygous mutant line bulked from a single family identified as a true-breeding short family in the M_4 (**Figure 7.2**). The "AF05" designation is subsequently used to refer to this homozygous dominant mutant line.

7.2.2. Seedling Photomorphogenesis

The most obvious characteristic of the *lip1* mutant is its de-etiolated appearance when grown in the dark. To further examine the similarity of the AF05 mutant to *lip1*, homozygous mutant seedlings were grown under monochromatic FR, R and B, in white light (WL), and in darkness. AF05 mutant plants showed a dark-grown phenotype not substantially different from WT plants (**Figure 7.4A**), indicating that the mutant phenotype is light-dependent and is thus quite unlike the *lip1* mutant. A small difference between WT and AF05 plants grown in the dark was occasionally observed (**Figure 7.4A**, note the slightly larger apical bud and more open apical hook in the AF05 seedling). This difference was increased by occasional exposure to green safelight, and may reflect a hypersensitivity to the safelight similar to that observed for the *hp-1* mutant of tomato (R.E. Kendrick, pers. comm.). **Figures 7.4B** and **7.5** show that the reduced elongation and increased leaf expansion of AF05 plants is strongly expressed under W, R and B, as it is under FR. This pattern of spectral sensitivity is also reminiscent of the *hp-1* mutant (Peters et al. 1992). PHYA overexpressors are also hypersensitive to both R and FR (McCormac et al. 1991, 1993), and the similarity between *hp-1* and transgenic tomato seedlings overexpressing the oat *PHYA3* gene has previously been noted (Kendrick et al. 1994) Thus the AF05 phenotype might result from a general enhancement of phytochrome

responses, as is thought to be the case for *hp-1* (Peters et al. 1992), or from selective enhancement of phyA responses.

The complete inability of phyA-deficient mutants to respond to FR (Dehesh et al. 1993, Reed et al. 1994, van Tuinen et al. 1995a, **Chapter 3**), together with the strong and apparently unique photolability of phyA (Quail 1991, Somers et al. 1991), indicates that phyA is the only phytochrome involved in mediation of responses to FR. Since AF05 is expressed under FR, it must therefore be hypersensitive to phyA at least. The fact that AF05 is also expressed under continuous, high fluence-rate R is not informative, as both phyA and phyB mediate de-etiolation responses under these conditions (**Figure 3.19**, McCormac et al. 1993). However, investigation of phyA- and phyB-deficient mutants has shown that the action of these phytochromes under R can be distinguished on the basis of either fluence rate or FR reversibility. A relatively consistent picture has emerged from study of *Arabidopsis*, tomato and pea mutants suggesting that phyB-dependent responses require relatively high fluences/fluence rates of R, and are reversible by FR, whereas phyA-dependentresponsesmay be induced by very low fluences/fluence rates of R or FR and are therefore not reversible by FR (Shinomura et al. 1994, 1996, Kerckhoffs et al. 1995, Reed et al. 1994, **Chapter 2, 3**). This distinction has been well-characterised for induction of anthocyanin synthesis and inhibition of hypocotyl elongation in tomato, where phyA and phyB1 respectively mediate low-fluence-rate and high-fluence-rate components of the response (Kerckhoffs et al. 1995). The *hp-1* mutant enhances both components (Kerckhoffs et al. 1995) and is therefore thought to act in the signal transduction pathway common to both phytochromes (Peters et al. 1992, Kendrick et al. 1994, Kerckhoffs et al. 1995).

In an attempt to distinguish between the action of phyA and phyB in the AF05 mutant, R/FR reversibility of de-etiolation in the mutant was examined. The results in **Figure 7.6A** show that leaflet expansion in AF05 was promoted to a much greater extent than WT by exposure to brief saturating R pulses given at 4-h intervals. However, the AF05 mutant retained a FR-reversible component similar in extent to that of WT and phyA-deficient *fun1-1* seedlings, and the increased effectiveness of the R pulses was largely due to an enhancement of the non-FR reversible component (**Figure 7.6**). The R/FR reversible component is clearly mediated by phyB, since it is absent in phyB-deficient mutants (McCormac et al. 1993, Reed et al. 1994, **Figure 3.11**) and selectively enhanced in phyB overexpressing lines (McCormac et al. 1993). The AF05 mutation therefore does not appear to substantially alter this response, suggesting that it may not affect phyB action but may specifically affect responses controlled by phyA. This distinguishes AF05 from the *hp-1*

mutant, in which both phyA- and phyB-mediated responses seem to be enhanced (Peters et al. 1989; Kerckhoffs et al. 1995).

7.2.3. Allelism Testing of AF05 with *fun1*

Given the physiological and genetic similarity of AF05 to phyA overexpressors, it is possible that the mutant might alter the levels or properties of phyA in some manner. As the next step in addressing this question, the genetic relationship between AF05 and the phyA-deficient *fun1-1* mutant was examined. In the case of recessive mutants, allelism is usually established by a mutant phenotype in the F_1 of a cross between the mutants in question. However, allelism between a dominant and a recessive mutant cannot be proven by classical means, and an allelism test cross can at best demonstrate close linkage of the two mutations. Such a cross is only useful in indicating non-allelism, if the two mutations are unlinked, or linked only distantly. In the case of two non-allelic, unlinked mutations, one dominant, one recessive, which affect the same phenotypic feature, the F_2 should segregate dominant:WT: recessive phenotypes in a 12:3:1 ratio, providing there is no complication resulting from interaction or incomplete dominance. To the extent that the mutations are linked, the proportion of WT (i.e. recombinant) types will decrease in favour of recessives. An F_2 population segregating *only* dominant and recessive types in a 3:1 ratio therefore indicates that the two mutants are closely linked, within a distance specified by the size of the population grown, and obviously includes the possibility that the mutants may be allelic.

The AF05 mutant was crossed to AF140 (*fun1-1*) and the F_1 and F_2 progeny grown under FR. **Figure 7.7** shows that as expected, the F_1 of the cross had an AF05 phenotype, in keeping with the near-complete dominance of the AF05 allele in internode 1 (**Figure 7.3B**). The F_2 population segregated 44 AF05-type:17 AF140-type plants, in clear accord with a 3:1 segregation ($\chi^2_{3:1}$=0.268, P>0.5). This result is therefore consistent with AF05 and *FUN1* loci being either closely linked or allelic. This same conclusion is supported by results from linkage crosses. Preliminary results from 74 F_2 plants from a linkage cross between AF05 and multi-marker L111 show AF05 to be linked to *K* (P<0.001, RCV=24.2±5.9) and to be on the verge of linkage with *A* (P≈0.1, RCV=36.0±7.3) (I.C. Murfet, pers. comm.). These recombination values are close to those found for *FUN1* with *K* (26.8±5.1) and with *A* (33.6±9.2) (**Figure 4.5**). Although larger populations must be grown to confirm these results, they do suggest that AF05 maps within about 5 units of *FUN1*.

7.2.4. Effect of AF05 on the Response to Photoperiod

Since the AF05 mutation had such a profound effect on seedling photoresponses, it was of interest to examine effects of the mutation on the flowering response to photoperiod. **Figure 7.8** shows that under a 24-h photoperiod (8 h of daylight with a 16-h extension with weak incandescent light) AF05 mutant plants, although shorter than WT, did not differ substantially from WT in terms of node of flower initiation (NFI), flower/leaf relativity index (FLR), time to first open flower (FT) or the total number of reproductive nodes (RN). In SD conditions however, AF05 plants flowered earlier, at a lower node, with a higher FLR, and produced substantially fewer reproductive nodes than WT plants. The AF05 mutant was therefore effectively day-neutral, flowering and senescing early under non-inductive conditions. This is also illustrated in **Figure 7.9**, which shows representative WT and AF05 plants grown under SD and LD. At 6 weeks after sowing, the AF05 plants in both photoperiods and the WT plant grown in LD have all flowered and terminated apical growth, whereas the WT plant in SD has yet to flower. The early-flowering and senescing phenotype of AF05 under SD is very similar to that of the previously described day-neutral mutants *sn* (Murfet 1971), *dne* (King and Murfet 1985) and *ppd* (Arumingtyas and Murfet 1994, Taylor and Murfet 1996). This is illustrated in **Figure 7.8** and **7.10**, in which the flowering responses and phenotype of AF05 are compared with those of *dne*. The AF05 phenotype is therefore the converse of that seen for the phyA-deficient *fun1-1* mutant (**Chapter 4**), in that it flowers early in non-inductive photoperiods rather than late in inductive photoperiods like *fun1-1*. This early-flowering phenotype is similar to that reported for transgenic *Arabidopsis* overexpressing phyA (Bagnall et al. 1995) and is consistent with the notion that the AF05 mutation may specifically enhance phyA activity.

One small but notable difference between WT and the day-neutral *dne* mutant is the fact that *dne* flowers 2-5 nodes earlier than the WT under LD (King and Murfet 1985). This is also true for the other day-neutral mutants *sn* and *ppd* (Murfet 1971, Arumingtyas and Murfet 1994, Taylor and Murfet 1996). This difference is thought to reflect the fact that the buried cotyledons are not exposed to light and synthesis of the flower inhibitor can still proceed in WT cotyledons even when the plant is grown in maximally inductive LD. This results in a slight delay of flowering, which can be reduced by exposure of the cotyledons to light within 4 days of sowing (Murfet and Reid 1974). This effect is also likely to explain the fact that the AF05 mutant flowered 2-3 nodes later than the *dne* mutant under both SD and LD despite showing the classical DN phenotype in all other respects (**Figure 7.8**). Exposure of AF05 cotyledons from day 4 after sowing significantly reduced this difference, lowering NFI from 15.6±0.1 to 13.8±0.2 in SD and from 14.3±0.2 to

13.2±0.2 in LD (n=6-8 in all cases), whereas exposure of *dne* cotyledons in a similar manner had no significant effect on NFI. This result indicates that the difference in NFI between AF05 and *dne* is due in part to an inhibitory influence of the unexposed AF05 cotyledons. This result also provides additional evidence that the early flowering phenotype of AF05 results from an enhanced response to light.

7.2.5. Phytochrome Levels in AF05

Taken together, the flowering and seedling photoresponses of AF05 are consistent with a phenotype that might be expected in a plant overexpressing phyA or hypersensitive to phyA action. The incomplete dominance of AF05 suggests an additional similarity to phyA overexpression, and the close linkage of AF05 with the phyA-deficient *fun1-1* mutant further increases the probability that AF05 may directly affect the *PHYA* gene. Therefore, phyA overproduction is probably the simplest among a number of possible explanations for the AF05 phenotype. To examine this possibility, the level of photoreversible phytochrome in AF05 was determined using *in vivo* spectrophotometry. This is a simple way of quantifying spectrally active phyA, since more than 95% of spectrophotometrically detectable phytochrome in etiolated pea seedlings is phyA (**Chapter 4**). **Figure 7.11A** shows that the phytochrome content of dark-grown AF05 seedlings was no higher than WT. However, when seedlings were exposed to continuous R, phytochrome appeared to be more slowly depleted in AF05 than in WT seedlings. Phytochrome depletion showed first-order kinetics in both the WT and AF05 seedlings, but the half-life in AF05 (154 min) was almost twice that seen in the WT (84 min, **Figure 7.11B**). Exposure times of longer than 6 h result in the accumulation of chlorophyll, and for this reason were not tested. However, after 6 h R, the AF05 mutant had 2.5 times the amount of phy (3.0±0.1 units) as WT (1.2±0.1 units). This probably represents a slightly larger difference in phyA level, since other phytochromes form a small proportion of the total pool remaining after prolonged R exposure (**Table 4.3**). However, since this method of measuring phytochrome content does not actually distinguish between the different phytochrome types, the possibility that AF05 may increase the level of a phytochrome other than phyA cannot currently be ruled out.

7.3. Discussion

Nature of the AF05 mutation

The AF05 mutant was isolated on the basis of exaggerated de-etiolation under FR, and subsequently shown to de-etiolate to a much greater extent than WT under R and B. A comparison between AF05 and various other mutants which display a somewhat similar phenotype is shown in **Table 7.1.** Light-dependence of the AF05 phenotype (**Figure 7.4A**) distinguishes it from the class of constitutively de-etiolated mutants which includes the *lip1* mutant of pea (Frances et al 1992). A light-dependent, light-hypersensitive phenotype similar to that of AF05 has previously been reported for the *hp-1* mutant of tomato and various transgenic phyA-overproducing lines. However, AF05 shows dominant inheritance (**Figure 7.2, 7.3, 7.4**) whereas *hp-1* is recessive. AF05 therefore represents a novel class of induced mutant, similar in phenotype and mode of inheritance to various transgenic phytochrome-overproducing lines.

The fact that AF05 is hypersensitive to FR suggests it does not specifically enhance phyB activity, since the effects of phyB overproduction are only seen under R (McCormac et al. 1993). However, both the spectral sensitivity (**Figure 7.5**) and the constitutively early flowering behaviour of AF05 (**Figure 7.9**) are consistent with a predominant effect on phyA activity, since phyA-overproducing transgenic lines are hypersensitive to both R (particularly at low fluence rate; McCormac et al. 1993) and FR (McCormac et al. 1991), and flower early in non-inductive photoperiods (Bagnall et al. 1995). The AF05 phenotype might therefore result from an enhancement of phyA activity alone, or enhancement of both phyA and phyB activity.

Physiological studies of phyA- and phyB-deficient mutants suggest that the actions of phyA and phyB in R-induced de-etiolation may be distinguished on the basis of R/FR reversibility or fluence rate. PhyB-deficient mutants lack responses which require relatively high fluence rates of R and are reversible by FR (McCormac et al. 1993, Shinomura et al. 1996), and the effects of phyB overexpression are apparently restricted to enhancement of these same responses (McCormac et al. 1993). In contrast, phyA-deficient mutants lack responses which require only low fluence rates and are not reversible by FR (Dehesh et al. 1993, Shinomura et al. 1996). However, certain results from phyA overproducing transgenic lines suggest that overexpressed phyA may in some circumstances mediate responses in a manner more characteristic of phyB, and may mask the action of the endogenous phyB. For example, overexpression of oat phyA in transgenic *Arabidopsis* appears to enhance both a FR-reversible response (for inhibition of hypocotyl

elongation) and a non-FR-reversible response (for apical hook opening and cotyledon expansion, Boylan and Quail 1991). Examination of R-pulse-induced apical hook opening in transgenic tobacco showed that overexpressed rice phyA enhances the hook-opening response to a FR pulse or, under some circumstances, the FR-reversible response to a sub-saturating R pulse (Schäfer et al. 1994). However, oat phyA overexpression in transgenic tomato appears to cause a selective enhancement of the low-fluence-rate component of R-induced anthocyanin biosynthesis (R.E. Kendrick, pers. comm.). These results show that it is difficult to reliably distinguish between the effects of phyA and phyB overexpression on R responses. However, in the case of etiolated AF05 seedlings, the selective enhancement of a non-FR-reversible response and the retention of a normal FR-reversible response (**Figure 7.6**) is consistent with an enhancement of phyA but not phyB function.

The finding that spectrophotometrically detectable phytochrome levels are elevated in AF05 seedlings (**Figure 7.11**) to an extent sufficient to account for the observed phenotype (Cherry et al. 1992) also lends support to the notion that AF05 may specifically affect the response to phyA, by virtue of an increased level of the photoreceptor in de-etiolating plants. In addition, the close linkage of AF05 and *fun1* (**Figure 7.7**) suggests that both mutations may affect the *PHYA* gene, further supporting the link between the AF05 phenotype and an effect on phyA level. This is obviously an attractive possibility and is perhaps the simplest explanation for the AF05 phenotype.

Further Investigation of the AF05 mutant

The different lines of evidence from genetic and physiological studies, and from measurement of phytochrome levels, all point toward AF05 being a mutation in the *PHYA* gene which increases the level of the expressed phyA protein in de-etiolating and de-etiolated seedlings. However, as a whole they provide only circumstantial evidence, as each individual line of evidence alone is inconclusive. In each separate area, further experiments are necessary. Perhaps most importantly, the use of a monoclonal antibody to quantify PHYA apoprotein in de-etiolating AF05 seedlings would establish whether the increase in level of spectrally active phytochrome (**Figure 7.11**) is indeed due to a specific increase in phyA level. Additional experiments might involve investigation of the effects of phyB deficiency on the AF05 phenotype, which would provide an indication of whether the AF05 phenotype is at all dependent on phyB activity. Another potentially useful way to discriminate between effects of AF05 on phyA level or phyA response would be to consider the dependence of the AF05 phenotype on the level of the phytochrome chromophore. In the case that the AF05 phenotype was related to the level of expression of the phyA molecule, a leaky chromophore-deficient mutant such as *pcd2* (**Chapter 6**)

might be expected to be epistatic to AF05, since the amount of chromophore would be the limiting factor in the response to light. Alternatively, if AF05 affected the inherent activity of phyA or affected signal transduction from the photoreceptor, then AF05 would presumably confer hypersensitivity to the small amount of active phyA produced in the presence of a leaky chromophore synthesis mutation, and the two mutants might therefore be expected to show an additive interaction. A distinction of this sort is apparent in a comparison of the epistasis relationships of the *hp-1* mutant and a transgenic phyA-overproducing line of tomato with the leaky chromophore-deficient *aurea* mutant. The *aurea* mutant is epistatic to the phyA overproducer (van Tuinen et al. 1996a), but shows an additive interaction with *hp-1* (Peters et al. 1989).

Assuming these investigations further support the connection between elevated level of phyA and the AF05 phenotype, the possible underlying reasons for this elevated level must then be considered. Since the level of phyA under any given light condition represents an equilibrium between synthesis and destruction, an elevated level and greater apparent stability of phyA could result from alteration to either process. The greater stability of phyA in AF05 could therefore result either from a failure to adequately repress expression of *PHYA* in the light, or from some defect which impaired the pathway required for normal phyA degradation. Neither of these explanations absolutely requires that the AF05 mutation directly affects the *PHYA* gene.

Distinguishing between possible effects of AF05 on *PHYA* expression or phyA degradation should be relatively straight-forward. The *PHYA* gene in pea is strongly down-regulated by light, in a R/FR reversible manner (Tomizawa et al. 1989, Furuya et al. 1991). Demonstration of a reduction in the P_{fr}-dependent repression of *PHYA* transcription would suggest that the AF05 mutation is likely to affect the *PHYA* promoter region in some manner. Analyses of the oat *PHYA3* promoter employing a transient expression system have identified a negative element, RE1, which appears to be necessary for P_{fr}-mediated down-regulation of *PHYA* expression, and which coincides with a region identified as a protein binding site in DNase protection assays (Bruce et al. 1991). Mutagenesis of this element results in constitutively maximal expression which is not influenced by phytochrome status (Bruce et al. 1991). Such a mutation in an endogenous *PHYA* gene *in planta* would be expected to confer a dominant phenotype. A motif homologous to RE1 is also present in the promoter of the pea *PHYA* gene (Sato 1988, Dehesh et al. 1994), and therefore one possibility is that AF05 is a mutation in the RE1-like element, preventing normal down-regulation of *PHYA* expression. Alternatively, it could result from a mutation in a gene coding for a putative trans-acting repressive factor which might bind to the RE1 element.

Should AF05 prove not to have a detectable effect on *PHYA* expression, the next possibility to be considered is that the mutation may alter the degradation of the phyA protein. Degradation is highly specific for P_{fr}, and occurs very rapidly after P_{fr} formation (Vierstra 1994). The earliest event following photoconversion is the aggregation or sequestering of phytochrome in the cytoplasm (Quail and Briggs 1979, McCurdy and Pratt 1986). Subsequent disappearance of P_{fr} occurs mainly from the sequestered population (Boisard et al. 1974, Mackenzie et al. 1975). Two mechanisms for phytochrome degradation have been proposed; the PEST mechanism (Rogers et al. 1986, Quail 1991) and the ubiquitin pathway (Shanklin et al. 1987, Vierstra 1994) but definitive evidence in support of either mechanism has yet to be demonstrated.

Regardless of the mechanism for phytochrome degradation, specific recognition of P_{fr} must take place at some point, since degradation is largely P_{fr}-specific. Nothing is currently known about the structural features of phytochrome required for sequestration. However, mapping of potential ubiquitination sites on the oat phyA molecule have identified a possible site between amino acids 742 and 790 (Shanklin et al. 1989), which, like the PEST sequence (amino acids 323-360) is more exposed in the P_{fr} than in the P_r form (Grimm et al. 1988). More detailed mapping of regions of the phy molecule important for P_{fr}-dependent degradation is obviously required. However, it is probable that such regions exist, and that mutation of critical residue(s) might decrease the rate of degradation and hence increase the equilibrium level of phyA. On the other hand, the energy requirement for sequestration (Quail and Briggs 1979) and the probable involvement of enzymatic activities in ubiquitin conjugation (Hershko and Ciechanover 1992) suggest that phytochrome degradation could also potentially be impaired by a mutation occurring outside the *PHYA* gene.

Thus, in the absence of any alteration to PHYA expression in AF05, it would become important to establish whether the mutation lies within the PHYA coding sequence, and also to examine processes potentially involved in degradation, such as sequestration and ubiquitination of phyA in the mutant.

Implications for Flowering in Pea

Mutants *sn*, *dne* and *ppd* have a very similar flowering phenotype to AF05, flowering and senescingearly in SD, and possessing a greatly reduced response to photoperiod (Murfet 1971b, King and Murfet 1985, Arumingtyas and Murfet 1994). However, flowering can be delayed under SD in these mutants by grafting onto a WT stock (Murfet 1971c, King and Murfet 1985, Taylor and Murfet 1996). This indicates that *sn*, *dne* and *ppd* are deficient in

the production of a substance normally required for inhibition of flowering under a SD regime. The early-flowering, day-neutral AF05 phenotype is therefore indicative of an abnormally low level inhibitor in SD. The fact that the phyA-deficient *fun1-1* mutant lacks the ability to respond to a photoperiod extension shows that it is phyA activity which normally shuts down inhibitor production (**Chapter 4**). It therefore appears that in WT plants grown under SD, phyA is not actively influencing flowering, whereas in the AF05 mutant, it is active. Although the lack of response to photoperiod suggests that inhibitor levels in AF05 are constitutively low, the promotion of flowering in AF05 by exposure of the cotyledons (which are normally buried 1-2 cm below the soil surface) indicates that the mutant does retain light-regulated inhibitor production to some extent. This is not the case for *sn*, *dne* and *ppd* mutants, which have a phenotype more consistent with a constitutive block in inhibitor production. This is illustrated diagrammatically in **Figure 7.12**.

It is possible that with a higher level of phyA activity, AF05 requires shorter daily exposures to light than WT for saturating promotion of flowering. It would be interesting to examine whether any delay of flowering occurred in AF05 plants grown under photoperiods shorter than 8 h. Alternatively, transfer of the AF05 mutation to a high-photoperiod-response background may make it clearer whether any *SN/DNE/PPD* activity is retained in mutant shoots exposed to normal (8 h) SD. Grafting experiments should be carried out to confirm that the early-flowering phenotype is indeed due to a reduction in inhibitor level. This could be most simply achieved by growing reciprocal grafts between AF05 and WT stocks under SD, similar to previous experiments with the *sn*, *dne* and *ppd* mutants. The fact that AF05 cotyledons appear able to produce inhibitor when buried suggests that grafting onto leafy stocks would be preferable to epicotyl/epicotyl grafts, since this would minimise the influence of the AF05 cotyledons.

Until the molecular basis for the AF05 phenotype is established, interpretation of flowering behaviour is somewhat limited. Nonetheless, when taken together with results obtained with the *fun1-1* mutant, the AF05 phenotype does support an important association between the level of phyA activity and the promotion of flowering, and emphasises the importance of phyA in mediation of the photoperiod response in pea. The way in which phyA-mediated promotion of flowering relates to the light/dark cycle is one of the more important questions to be answered next. It has been shown that for some LDP species, promotion of flowering by FR exhibits a rhythmic sensitivity, and that this rhythm is itself subject to phase-shifting in response to FR (Deitzer et al. 1982). This rhythm runs in the light, and has been suggested to be related to the requirement for long photoperiods (Vince-Prue and Takimoto 1987). Some LDP species also show a rhythmic

response to R, which is suspended in continuous light and relates to measurement of a critical night-length. A detailed study examining the effect of night-breaks and supplementary FR in WT, fun1 and AF05 mutants may give a more specific picture of the way in which phyA contributes to the photoperiodic response. Investigation of the effect of photoperiod on *PHYA* expression in WT and AF05 plants may also be useful in addressing this question. Also important is the question of whether inhibitor production is influenced by both phyA and phyB, or by phyA alone. Finally, identification of the inhibitory influence itself will be a significant advance in understanding of the control of photoperiodism in pea.

7.4. Methods

The AF05 mutant was identified in an M_2 population of EMS-mutagenised cv. Torsdag grown under 8 μmol m^{-2} sec^{-1} FR (see **Chapter 4**). This mutant plant yielded WT and mutant segregates in the M_3, consistent with it being heterozygous for a dominant mutation. A true-breeding mutant family was identified in the M_4, and used for crossing and for generation of a homozygous dominant mutant line, subsequently reffered to as AF05 (**Figures 7.4 - 7.11**). The origins of the *lip1* mutant from cv. Alaska and the *dne* mutant from cv. Torsdag have been described (Frances et al. 1992, King and Murfet 1985).

Plants used in the experiments shown in **Figure 7.6** and **7.11** were grown in water-saturated, drained vermiculite, in growth cabinets at 25°C. Standard RIKEN light sources were used in these experiments. All other plants were grown in standard Hobart pea potting mix, either in the phytotron (**Figure 7.8, 7.9, 7.10**) or in growth cabinets at 20°C, using standard Hobart light sources. Where necessary, standard green safe-light was sparingly used for manipulation of etiolated seedlings. *In vivo* spectrophotometry was performed as described in **Chapter 2**.

Table 7.1. Comparison of mutants and transgenic phytochrome-overexpressing lines showing an "exaggerated de-etiolation" phenotype under red light.

Character	Mutant / transgenic line				
	lip1	*hp-1*	phyB^{++}	phyA^{++}	AF05
expressed in R	+ [5]	+ [2,4]	+ [11]	+ [11]	+
expressed in FR	+ [5]	+ [4]	- [11]	+ [8,10]	+
expressed in D	+ [1]	- [2,4]	- [7,11]	- [8,10]	-
R pulse response					
- FR reversible		+ [2,4]	+ [11]	+ [10]	-
- non-FR-reversible		+ [2,4]	-	+ [10]	+
R fluence rate					
- low		+ [3]	+ [11]	+ [11]	?
- high		+ [3]	+ [11]	+ [11]	+
inheritance	R [1]	R [2]	D [7]	D [6,10]	D
elevated phy	- [1]	- [4]	+ [7]	+ [6]	±
interaction with chromophore deficiency		A [2]	?	H [9]	?

R-recessive, D-dominant, A-additive, H-hypostatic. Data from [1]Frances et al. 1992, [2]Peters et al. 1989, [3]Kerckhoffs et al. 1995, [4]Peters et al. 1992, [5]Weller JL, unpublished, [6]Boylan and Quail 1989, [7]Wagner et al. 1991, [8]McCormac et al. 1992, [9]van Tuinen et al. 1996a, [10]Boylan and Quail 1991, [11]McCormac et al. 1993.

Figure 7.1. Phenotype of the AF05 mutant under FR. For comparison, the *lip1* mutant is also shown, together with its progenitor, cv. Alaska (ALA). All seedlings are 10 d old. Growing conditions; FR, 8 μmol m^{-2} sec^{-1}, 20°C.

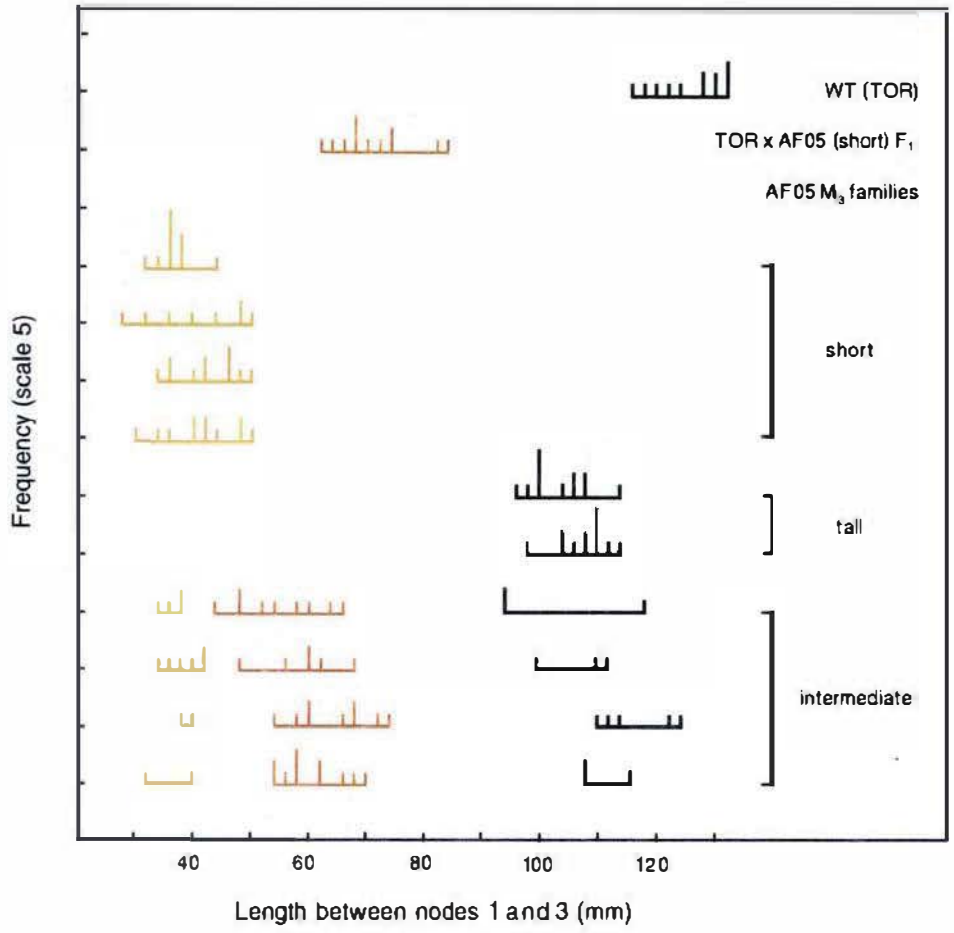

Figure 7.2. Genetic analysis of the AF05 phenotype. M_4 progeny were grown from representative short, intermediate and tall segregates in the progeny (M_3) of the original AF05 mutant (M_2). F_1 plants of the cross TOR x AF05 (short M_3 segregate) are also shown for comparison. Black and orange bars represent plants with WT and mutant phenotype, respectively. Growing conditions; R, 20 μmol m^{-2} sec^{-1}, 20°C.

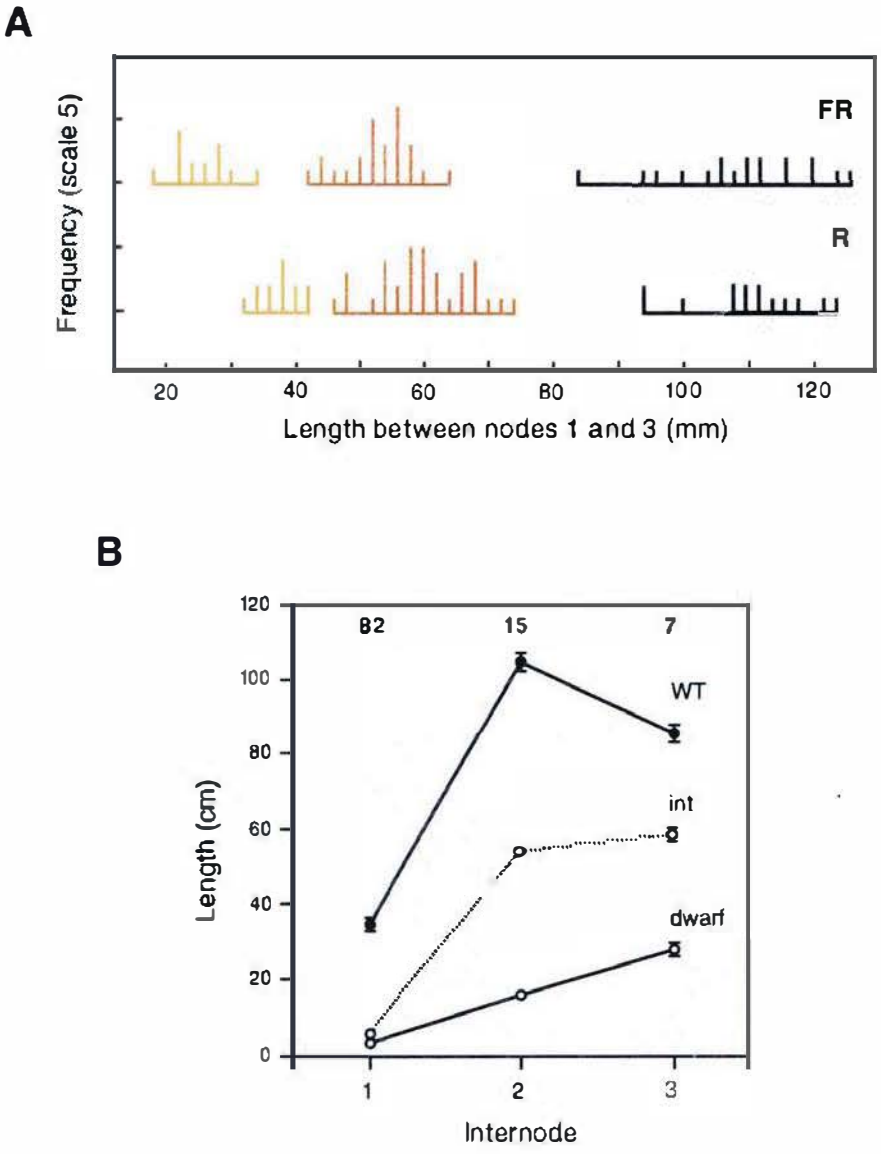

Figure 7.3. Genetic analysis of the AF05 phenotype. **A.** F_2 progeny from a cross between TOR and a short (homozygous mutant) segregate in the M_3 progeny of the original AF05 mutant were grown under FR (8 μmol m^{-2} sec^{-1}) and R (20 μmol m^{-2} sec^{-1}) at 20°C. Black and orange bars represent plants with WT and mutant phenotypes, respectively. **B.** Mean internode lengths for dwarf (n=14), intermediate (n=26), and tall (n=18) segregates in the F_2 progeny grown under FR. Numbers in bold at the top of the graph indicate the degree of dominance (%) of the mutant over the WT allele, based on the mean length values for each internode.

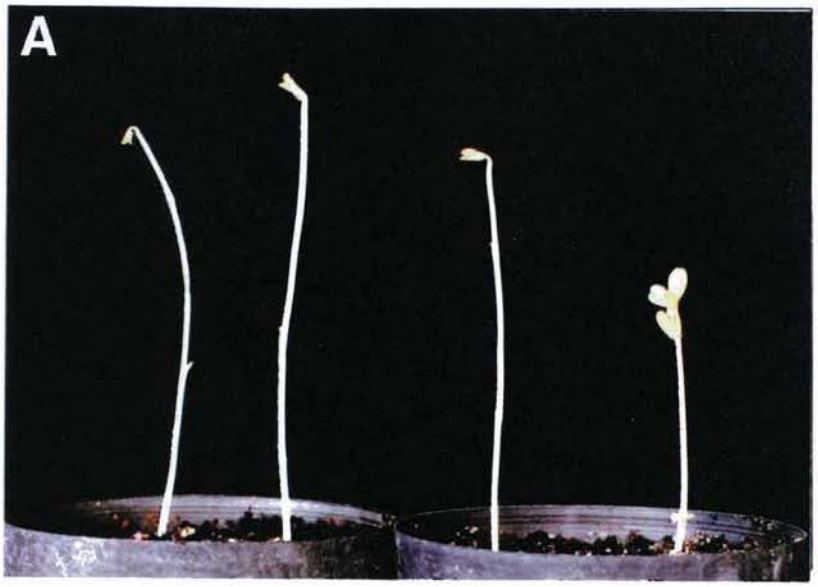

Figure 7.4. Phenotype of the AF05 mutant. **A.** Representative cv. Torsdag (TOR), AF05, cv. Alaska (ALA) and *lip1* seedlings grown in complete darkness for 7 days from sowing. **B.** Representative TOR and AF05 seedlings grown under FR (8 μmol $m^{-2}sec^{-1}$), R (20 μmol $m^{-2}sec^{-1}$), WL (150 μmol $m^{-2}sec^{-1}$) or B (10 μmol $m^{-2}sec^{-1}$) at 20°C for 8 days from sowing.

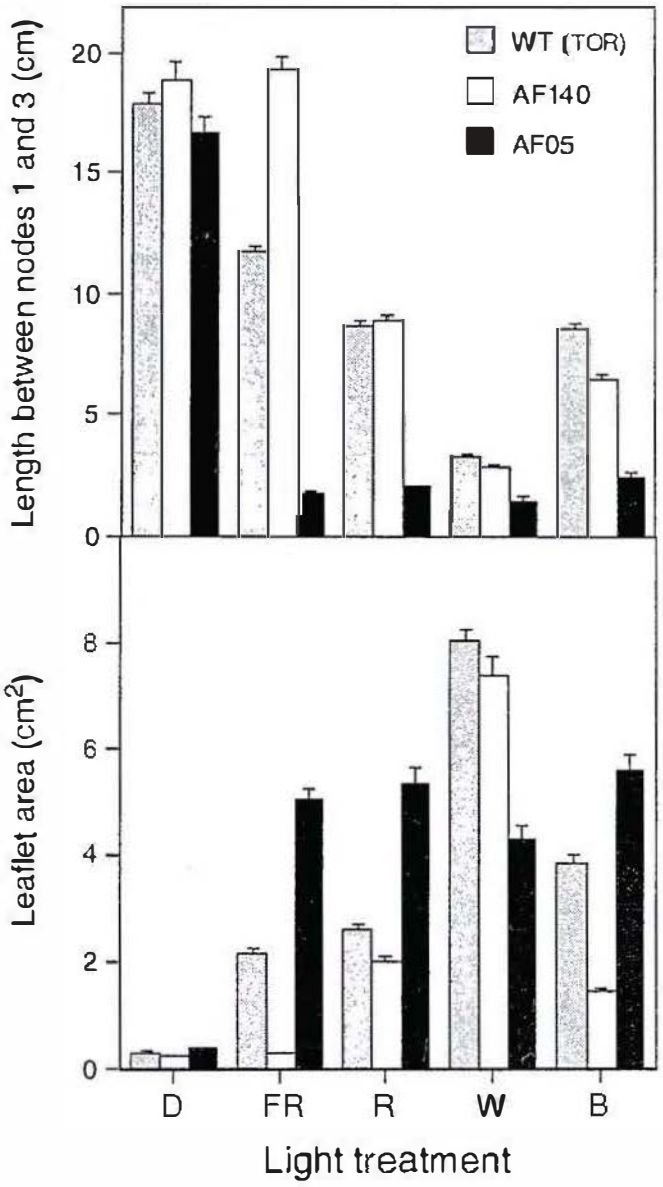

Figure 7.5. Phenotype of the AF05 mutant under monochromatic light. WT cv. Torsdag (TOR), AF05 and *fun1-1* mutant seedlings were grown in the dark (D) or under FR (8 μmol m^{-2} sec^{-1}), R (20 μmol m^{-2} sec^{-1}), WFL (150 μmol m^{-2} sec^{-1}) or B (10 μmol m^{-2} sec^{-1}) at 20°C. Leaflet area was estimated as the product of the length and width of a single leaflet from the first true foliage leaf (leaf 3). Error bars represent SE, n=8-12.

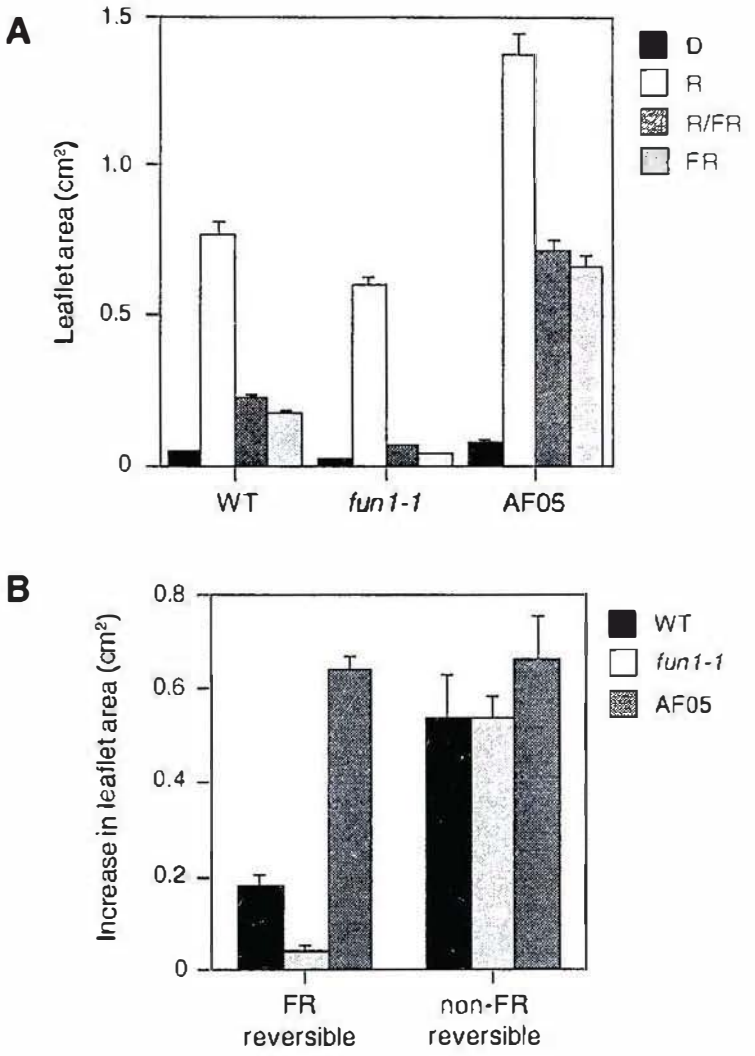

Figure 7.6. R/FR reversibility of de-etiolation in WT and AF05 seedlings. The phyA-deficient *fun1-1* mutant was included for comparison. Plants were given saturating pulses of R (17 μmol m^{-2} sec^{-1}, 10 min), FR (12 μmol $m^{-2}sec^{-1}$, 15 min), or R followed by FR (R/FR) at 4-h intervals for 10 d after sowing, or maintained in complete darkness (D), at 25°C. **A.** Leaflet area, estimated as the product of the length and width of a single leaflet from the first true foliage leaf (leaf 3). **B.** Comparison of FR-reversible (R-R/FR) and non-FR-reversible (R/FR-D) components of the response to R pulses shown in A. Error bars represent SE, n=8-10.

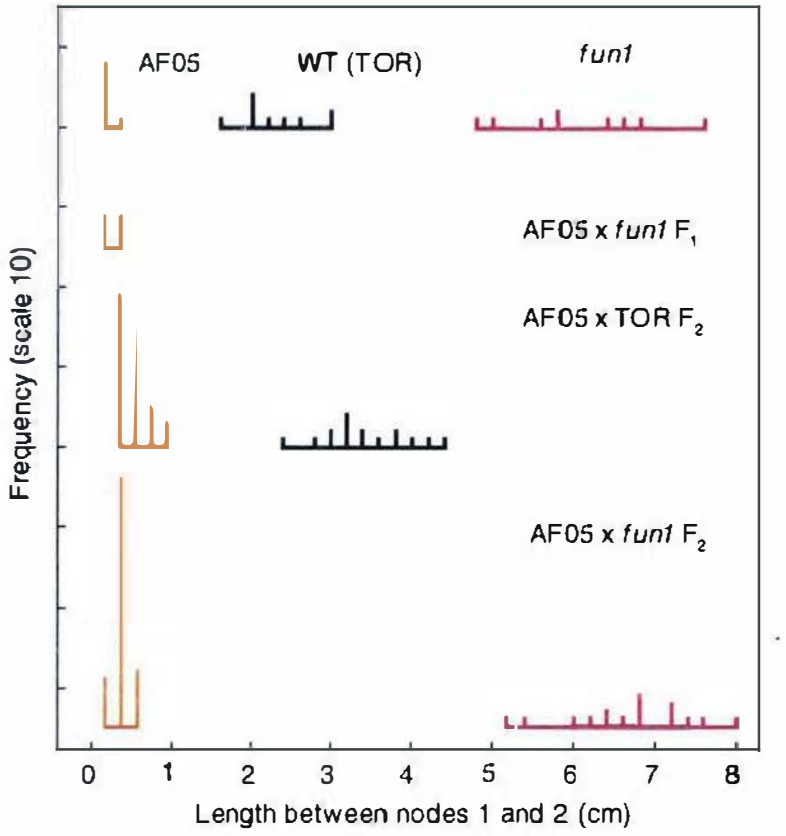

Figure 7.7. Allelism testing and interaction of AF05 with *fun1*. A true-breeding (homozygous) AF05 mutant segregate was crossed to the *fun1-1* mutant, and F_1 and F_2 progeny of this cross were grown under FR (8 μmol m^{-2} sec^{-1}) at 20°C. Data from F_2 progeny of a cross between AF05 and wild-type (WT) cv. Torsdag (TOR) are included for comparison. Black and orange and purple bars represent plants with WT, AF05 and *fun1-1* mutant phenotypes, respectively.

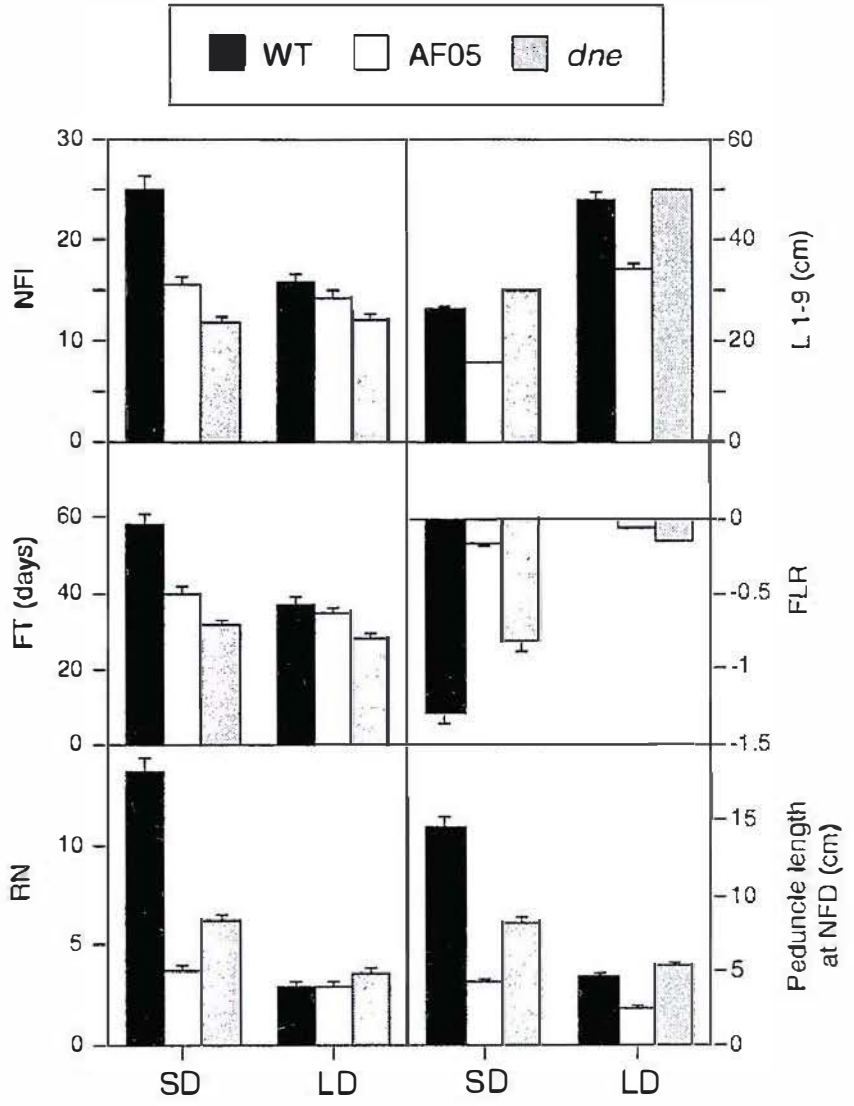

Figure 7.8. Responses of the AF05 mutant to a photoperiod extension. Plants were grown from sowing in an 8-h photoperiod of natural daylight with (LD) or without (SD) a 16-h extension given as weak incandescent light (3 μmol m^{-2} sec^{-1}). The day-neutral mutant *dne* was included for comparison. NFI - node of flower initiation, FT - time to first open flower, FLR - flower/leaf relativity index, L 1-9 - stem length between nodes 1 and 9, RN - number of reproductive nodes. Bars indicate SE, n = 6-8.

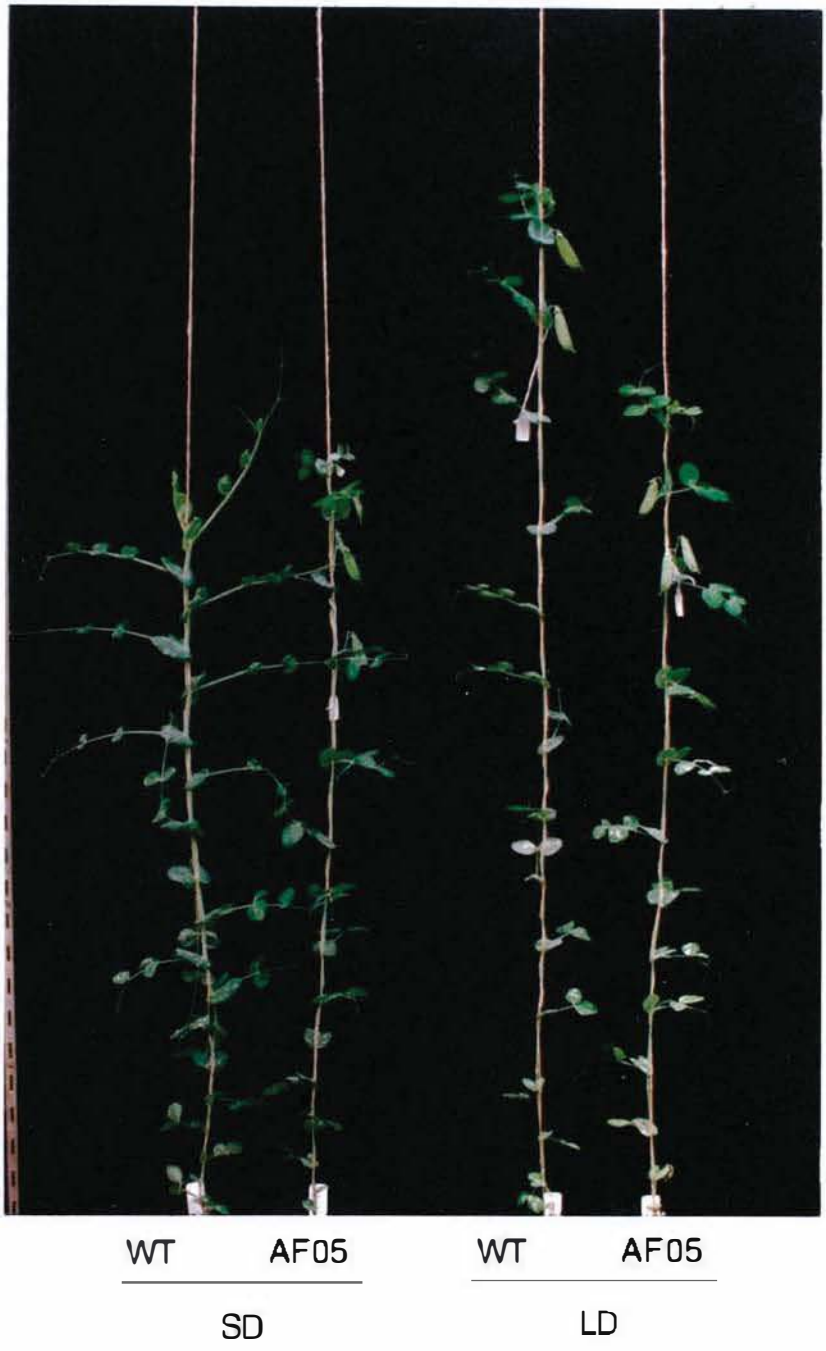

Figure 7.9. Response of the AF05 mutant to a daylength extension. Plants were grown for 6 weeks from sowing in an 8-h photoperiod of natural daylight with (L◗) or without (S◗) a 16-h extension given as weak incandescent light (10 μmol $m^{-2}s^{-1}$).

Figure 7.10. Comparison of the phenotypes of the AF05 and *dne* mutants. Plants were grown for 6 weeks from sowing in an 8-h photoperiod of natural daylight. Both the AF05 and *dne* mutants have flowered and apical growth has ceased, whereas the wild-type cv. Torsdag plant (WT) has yet to flower.

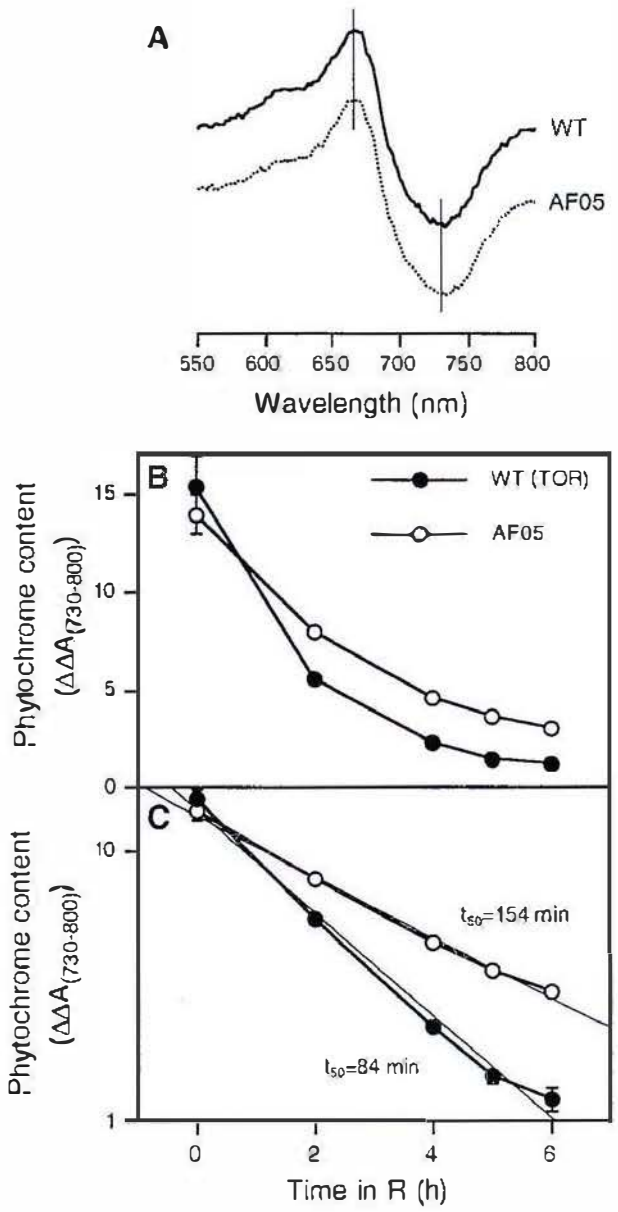

Figure 7.11. Comparison of phytochrome content in wild-type (WT) cv. Torsdag (TOR) and AF05 mutant seedlings using *in vivo* spectrophotometry. **A.** Difference spectra for *in vivo* phytochrome phototransformation (P_{fr}-P_r) in 5-day-old etiolated seedlings. B. Kinetics of phytochrome depletion following transfer of 5-day-old etiolated seedlings to R (17 μmol $m^{-2}s^{-1}$). Each point is the mean of three replicates (from separate plantings) with three determinations per replicate. C. Log-linear plot of the data from panel B. Values shown are half-lives for phytochrome depletion, calculated from the fitted curves shown in C. Bars indicate SE; where not visible, bars are smaller than the plot symbols.

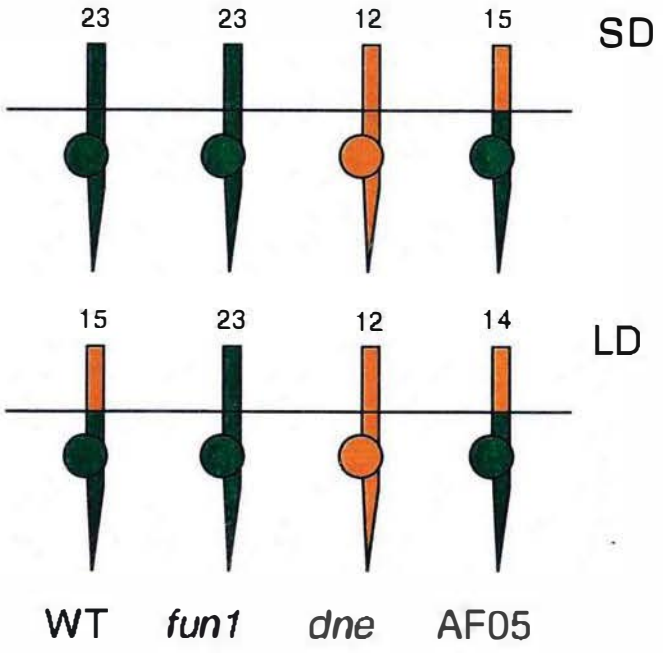

Figure 7.12. Scheme relating the flowering phenotype of WT, *fun1*, *dne* and AF05 plants to inferred sites of inhibitor production. Diagrammatic plants are shown as a root-shoot axis with cotyledons. Horizontal lines represent the soil surface. Sites of inhibitor production are shown in green, sites with little or no inhibitor production are shown in yellow. The value given above each plant is the approximate flowering node, under standard phytotron SD (8-h) and LD (24-h) conditions.

8. Concluding Discussion

This study has employed a genetic approach to investigate the influence of light on development of the garden pea, and has identified several genes required for normal light-responsiveness. Mutants at the *LV* locus show reduced sensitivity to R and WL, and are deficient in phytochrome B, while mutants at the *FUN1* locus are insensitive to FR and are deficient in phytochrome A. Mutants at the *PCD1* and *PCD2* loci have reduced sensitivity to both R and FR, and impair steps in the synthesis of the phytochrome chromophore, phytochromobilin. Finally, mutant line AF05 shows many characteristics of elevated phyA level or exaggerated response to phyA. These characteristics result from a dominant mutation linked to *FUN1*. Study of these mutants has yielded results which are largely consistent with results from investigation of similar mutants in other species. Together, these results provide a coherent model for the way in which various photoreceptors interact to control development in pea. This chapter presents a overview of the results obtained, and a brief discussion of their relationship to other similar work.

8.1. Phytochrome Synthesis

The demonstration that the chromophore-deficient *pcd1* mutant blocks the conversion of heme to BV IXα represents the first instance in which chromophore deficiency has been shown to result from impairment of a specific step in PΦB synthesis. In addition it has also provided important confirmation of the enzymatic nature of the heme to BV conversion, which could not previously be assumed (Terry et al. 1993). The isolation of the *pcd2* mutant, deficient in PΦB synthase activity, is also significant. Although PΦB synthase has been previously characterized, it has not been clear from these studies whether it is required for the isomerization of of 3Z-PΦB to 3E-PΦB. The *pcd2* mutant provides a useful tool for examining this question of whether this isomerization is achieved by PΦB synthase, by an additional enzyme activity ("PΦB isomerase", Terry et al. 1993), or via a non-enzymatic mechanism.

The *pcd1* and *pcd2* mutants has also provided important new information about the phenotypic effects of chromophore-deficiency. Chromophore-deficient mutants in other species are all reported to have a pale yellow-green appearance. This chlorosis has been suggested to result either from impairment of tetrapyrrole synthesis at a point of the pathway common to both chlorophyll and PΦB synthesis, or from reduced phytochrome induction of Chl synthesis. However, the *pcd1* and *pcd2* mutants provide evidence against both these explanations. The *pcd1* mutant, which shows strong chlorosis (**Figure 5.1**), is

blocked after the branch point for Chl and PΦB synthesis, indicating that development of the yellow-green phenotype does not require direct impairment of Chl synthesis. In contrast, the relatively normal chlorophyll content of the *pcd2* mutant (**Figure 6.7**) indicates that a strong deficiency in active phytochrome is not sufficient to confer the chlorosis observed in *pcd1*. An alternative explanation for the phenotype is suggested, involving an accumulation of heme as a result of impaired heme to BV conversion (or in some species, BV to PΦB conversion also). This may have inhibitory and perhaps even mildly toxic effects on Chl synthesis and the development of the photosynthetic apparatus. The *pcd1* and *pcd2* mutants will provide a useful means of further exploring this question.

8.2. Phytochrome and De-Etiolation

Physiological experiments with phyB- and phyA-deficient mutants (**Chapter 3** and **4**) have defined distinct roles for these phytochromes in control of pea seedling de-etiolation under white and monochromatic light. The results can be summarised as follows;

1. The effects of phyB deficiency are observed primarily under R, with a small effect under B, and no effect under FR (**Figures 2.8, 3.6, 3.19**).
2. In contrast, the effects of phyA deficiency are observed primarily under FR, and to a lesser extent under R and B (**Figure 3.6, 3.19**). However, in the absence of phyB, phyA deficiency also has a large effect under R (**Figure 3.19**).
3. In the absence of both phyA and phyB plants respond minimally if at all to R or FR, but retain substantial sensitivity to B (**Figure 3.19**)
4. The action of phyA and phyB under R can be distinguished on the basis of FR-reversibility or fluence-rate threshold (**Figures 2.11, 3.10, 3.19**).
5. Effects of phyB can under some circumstances be reversed by FR, or reduced by addition of FR (**Figures 2.11, 2.12, 2.13**).
6. phyA promotes elongation under B (**Figure 3.19**).

Results **1** and **2** suggest that the effects of light on pea seedling development can be largely explained in terms of the co-action of photoreceptors with overlapping spectral sensitivities, as shown in **Figure 8.1A**. The effects of FR are mediated by phyA alone, the effects of R are mediated by phyA and phyB, and the effects of B mediated by phyA, phyB and a blue-light photoreceptor. These loosely defined spectral sensitivities of phyA and phyB are in keeping with various results obtained in *Arabidopsis* from both mutants and transgenic plants, which indicate that phyA is active over the entire spectrum from 350 to 750 nm, whereas inductive effects of phyB are restricted to the red

(McCormac et al. 1993, Liscum and Hangarter 1993, Shinomura et al, 1996) and, to the lesser extent, the blue regions (Chory 1992). In pea, phyA appears to have a relatively important role in R-induced de-etiolation, in comparison to *Arabidopsis* (Reed et al. 1994). As a result, the mutual compensation of phyA and phyB under R in pea (Result 3) is somewhat more clear than in *Arabidopsis*. This is likely to be a quantitative rather than a qualitative difference between the species however, and could result from relatively small differences in the regulation of phyA level. Identification of any role for phytochromes other than phyA and phyB under R must await a more detailed characterisation of double mutants deficient in both phyA and phyB.

With respect to the mediation of B-induced de-etiolation, the two species appear to differ to a greater extent. A recent preliminary report indicates that in *Arabidopsis*, the CRY1-mediated inhibition of elongation depends on the presence of either phyA or phyB (Ahmad and Cashmore 1996). In pea this does not appear to be the case, since elongation is still strongly inhibited by B when both phyA and phyB are absent (Result 3). Although this difference needs to be more thoroughly explored, it raises the question of the extent to which interactions among the photoreceptors differ between species. A promotory effect of phyA on stem elongation under B (result 6) has not previously been reported, and will also require further investigation. One possible explanation is that $P_{fr}A$ and P_rA have opposing effects on stem elongation, and that the B conditions used in this study reveal a promotory effect of P_rA. The possibility has recently been discussed that in the case of both phyA and phyB, P_r and P_{fr} may both be active, and may have opposite effects (Quail et al. 1995, Smith 1995).

Results 3-5 also address the differences between phyA and phyB. As in *Arabidopsis*, red light responses mediated by phyA in pea require a much lower fluence/fluence rate than those mediated by phyB, suggesting that $P_{fr}A$ may be more active per mole than $P_{fr}B$. These observations are thus consistent with recent explanations of the difference in photosensory specificity of phyA and phyB (e.g. Furuya and Schäfer 1996). Domain-swapping experiments with transgenic phyA and B in *Arabidopsis* have suggested that phyA and phyB share interchangeable C-terminal regulatory regions, with their photosensory specificity and differential light-lability conferred by their N-terminal domains (Wagner et al. 1996a). These results suggest that phyA and phyB may have a shared primary reaction partner. However, the isolation of additional loci specifically required for de-etiolation under FR (Whitelam et al. 1993), and the ineffectiveness of micro-injected phyB in the induction of anthocyanin biosynthesis (Kunkel et al. 1996) suggest that the functions of phyA and phyB may not overlap completely, and therefore imply the existence of more than one primary mechanism of phytochrome action.

8.3. Phytochrome and Green Plant Photoresponses

Photoresponses in the de-etiolated pea seedling also result from the interaction of phyA and phyB. PhyB inhibits elongation and promotes de-etiolation under light at high R:FR, and is less effective under conditions which reduce the phytochrome equilibrium (**Figure 3.12, 3.13**). At low R:FR, where phyB is less active, a contribution from phyA is also revealed (**Figure 3.12, 4.9**). These observations are again consistent with current understanding of phytochrome action in the green plant (Smith 1995).

However, at some point in the development of a WT pea plant under long day conditions, the effect of phyA action on stem elongation reverses, and a phyA-mediated promotion of stem elongation becomes apparent (**Figure 4.9**). Whether this replaces, or merely overrides the earlier inhibitory effects is not clear. Such a response has not previously been reported for phyA-deficient mutant in other species. This promotory effect of phyA on stem elongation is clearly associated with other effects such as the promotion of flowering and senescence (**Table 4.3**). PhyA therefore appears to mediate the previously described FR-HIR for promotion of flowering in pea (Reid and Murfet, 1977), as also appears to be the case in *Arabidopsis* (Johnson et al. 1994). PhyA appears to act by down-regulating of the production or transport of a graft-transmissible flower-inhibitory influence. This inhibitor has many other effects in addition to inhibition of flowering (Murfet 1982, 1985, **Table 4.3**). PhyA action is therefore dependent on the presence of the *DNE* gene, which is required for normal inhibitor production under non-inductive photoperiods (King and Murfet 1985, **Figure 4.19, 4.20**). These observations indicate the presence of two distinct pathways for phyA action (**Figure 8.1B**). Although branch-points in the transduction of signals from phyA have previously been identified (Bowler et al. 1994, Johnson et al. 1994, Barnes et al. 1995), the present study provides the clearest distinction to date between the effects of phyA on de-etiolation and on photoperiod detection. This study is also the first to demonstrate the effects of a specific phytochrome on a graft-transmissible regulator of flowering.

In contrast to phyA, phyB in pea has an inhibitory influence on flowering in plants grown under non-inductive conditions (**Figure 3.15**). This effect of phyB has yet to be related to any of the response modes identified from previous physiological studies of flowering in LDP. It is also not yet clear whether this effect is also achieved through regulation of flower inhibitor production.

Although phyA and phyB are both required for normal development throughout the life of the plant (**Figure 3.2, 4.3**), mutants deficient in either photoreceptor still achieve de-

etiolation and are relatively vigorous when grown under standard glasshouse long-day conditions. However, a deficiency in both phytochromes severely impairs the normal development of plants grown under the same conditions (**Figures 4.15, 4.16**). This indicates a fairly high degree of mutual compensation between phyA and phyB. Although the possibility of leakiness cannot be discounted, the weak de-etiolation seen in the double mutant does suggest a limited contribution from an additional photoreceptor. Furthermore, since the double mutant phenotype grown in LD is considerably more severe than that of a phyB-deficient mutant grown in SD, it implies that phyA is active in SD and can partially compensate for the loss of phyB.

8.4. Future Prospects

The work presented in this thesis has established a broad and solid basis for further investigations into the photocontrol of development in pea. Many of the specific questions to be addressed have been discussed in earlier chapters. However, it can be said that the next and most important general aim should be to identify the molecular bases of the lesions in all the mutants described. This should be relatively straight-forward in the case of the *lv* and *fun1* mutants given the availability of *PHYA* and *PHYB* clones in pea (Sato 1988, Beauchamp 1996). Molecular characterisation of the *pcd1* and *pcd2* mutants may have to await identification and cloning of the corresponding genes from *Arabidopsis* or tomato, a process which is underway (MJ Terry, pers. comm.). Of the many physiological questions which could be addressed in future work, among the most important involve (a) the use of a confirmed *phyA phyB* null mutant to identify possible roles for other phytochromes in de-etiolation and throughout development, (b) more detailed investigation of flowering physiology in order to understand the way in which phytochromes A and B interact with the circadian rhythm to mediate photoperiodic responses, and (c) examination of interactions of phytochrome-deficient mutants with other genes involved in the control of flowering. Results from such studies will help in the development of specific screens to identify additional mutants affecting subsets of phytochrome-controlled responses.

As a model species for developmental genetics, pea will always be somewhat limited in certain important respects relative to *Arabidopsis* and tomato. However, this thesis has demonstrated that for certain specific questions, the use of pea may be equally suitable, and may even be preferable. There is no doubt that a continued effort to extend the number and taxonomic diversity of model species will greatly benefit further understanding of photomorphogenesis.

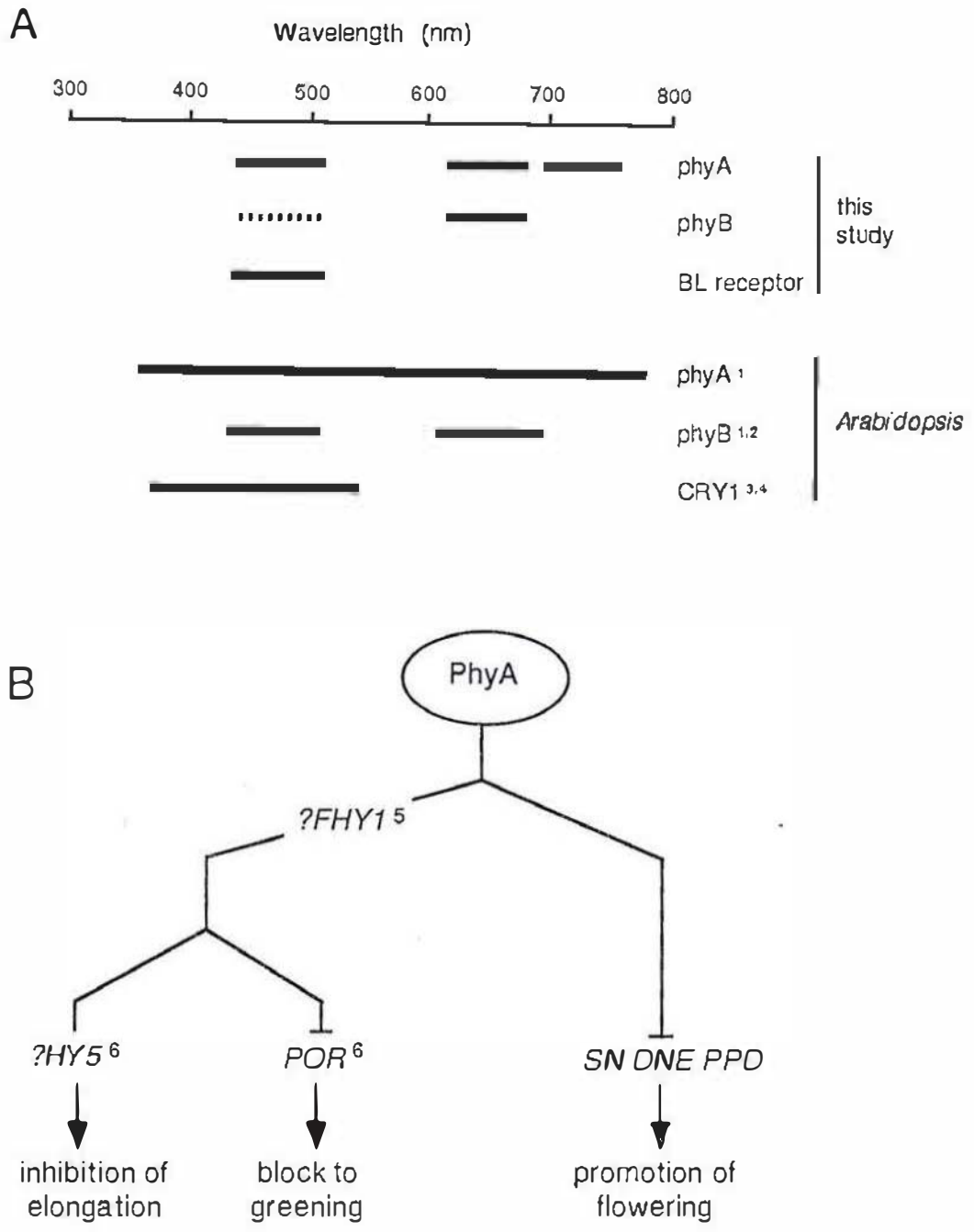

Figure 8.1. A. Comparison of spectral sensitivities for phyA-and phyB-controlled responses in pea and *Arabidopsis*. B. Diagram showing putative branch-points in the transduction of responses to phyA. Results taken from the present study and from [1]Shinomura et al. 1996, [2]Chory 1992, [3]Ahmad and Cashmore 1993, [4]Ahmad et al. 1995, [5]Johnson et al. 1994, [6]Barnes et al. 1996.

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Internode length in *Pisum*. Two further *lv* mutants.

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The mutant alleles of four major internode length genes in pea, *La*, *Cry*, *Sln* and *Lv*, have now been shown to result in a phenotype with elongated internodes. The double mutant *la cry*^{s} has long, thin internodes and a phenotype known as slender (1,2). The *la cry*^{s} slender plants behave as if saturated with gibberellins (GAs), and the slender phenotype is expressed even in genotypes grossly deficient in GAs (6). More recently, a new mutant with elongated internodes and a phenotype similar to *la cry*^{s} slender plants, at least in regard to the lower internodes, was shown to result from the *sln* mutation (9). Plants homozygous for *sln* have elevated levels of C_{19} gibberellins. It is suggested (9) that the *sln* mutation may block catabolism of GA_{20} in the seeds and that this results in elevated levels of GA_1 in the young seedling.

Plants homozygous for the mutant allele *lv* have elongated internodes, a semi-etiolated appearance, and an enhanced response to applied GA_1 (5,8). The *lv* allele is thought to block the action of light-stable phytochrome (5), and differences between *Lv* and *lv* plants are maximised under a regime of continuous light from cool white fluorescent tubes (10) since this light source provides only a negligible level of far-red light.

We report here on inheritance and allelism tests with two further pea mutants, L80m and Wt10895, with elongated internodes. Both mutants showed monogenic recessive inheritance and proved to be allelic with *lv*.

Materials and Methods

The elongated mutant L80m arose spontaneously in our stocks of line L80 (Lamm L30). The elongated mutant Wt10895 was obtained at Wiatrowo Plant Breeding Institute by Prof Dr W.K. Swiecicki following treatment of the dwarf cultivar Paloma (Wt3527) with 200 r Nf (fast neutrons)/0.014% NEU (nitroso ethyl urea). These two elongated mutants were tested for allelism with *lv* by crossing with NEU3 (*lv*; 8) an elongated mutant obtained by Dr T.A. LaRue from cv. Sparkle. Hobart lines L2 (Lamm line 2) and L85 were also used in crosses. Lines 2 (*le la Cry Lv*; 2,4), 80 (*le La Cry Lv*; 3), 85 (*le La cry*^{c} *Lv*; 4) and Sparkle (*le La cry*^{c}*Lv* ; 7) are all phenotypically dwarf.

The plants were grown in 14 cm slim line pots in a 1:1 (v:v) mixture of vermiculite and 10 mm dolerite chips topped with 4 cm of sterilised peat-sand potting mixture. Nutrient was supplied once a week in the form of Total Growth Nutrient (R and D Aquaponics, Sydney). The measurements in Figs 1, 2 and 4 were obtained from plants grown in growth cabinets under continuous light from cool white fluorescent tubes (Figs 1 and 4, 90 μmol m^{-2} s^{-1} and Fig. 2, 6 μmol m^{-2} s^{-1}) at a constant temperature

(Figs 1 and 4, 20°C, and Fig. 2, 17.5°C). These conditions were used during the early stages of growth to screen the genotypes. After separation was effected (usually 2-3 weeks), the plants were transferred to normal glasshouse conditions for growth to maturity. The data in Fig. 3 come from plants grown under an 8 h photoperiod (8 h daylight, 16 h dark; temperature 18-22/16°C). Node counts commenced from the first scale leaf as node 1.

Results and Discussion

The data in Fig. 1 were obtained from several progenies segregating L80-type (dwarf) and L80m-type (elongated) plants. The observed numbers of 85 dwarf and 35 elongated segregates are in good accordance with a 3:1 ratio ($\chi^2 = 1.11$, $P > 0.2$) and

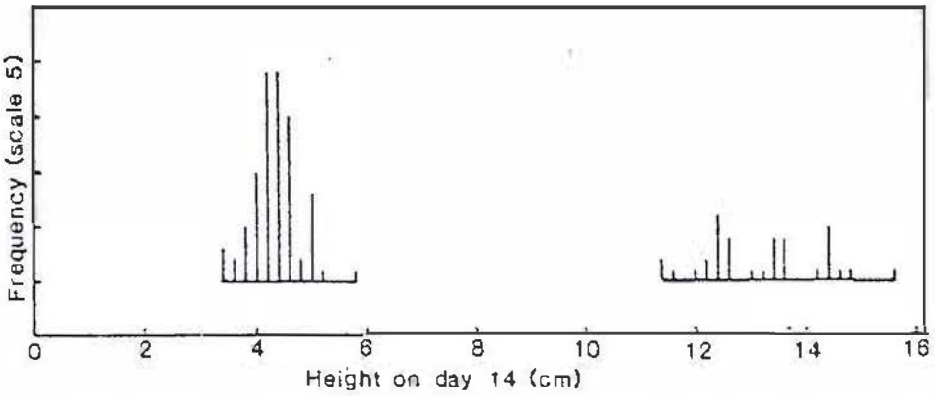

Fig. 1. Distribution of height (soil surface to shoot apex) at day 14 for line 80 progenies segregating for dwarf (L80) and elongated (L80m) types. Conditions: continuous white fluorescent light (90 µmol m^{-2} s^{-1}) at 20°C.

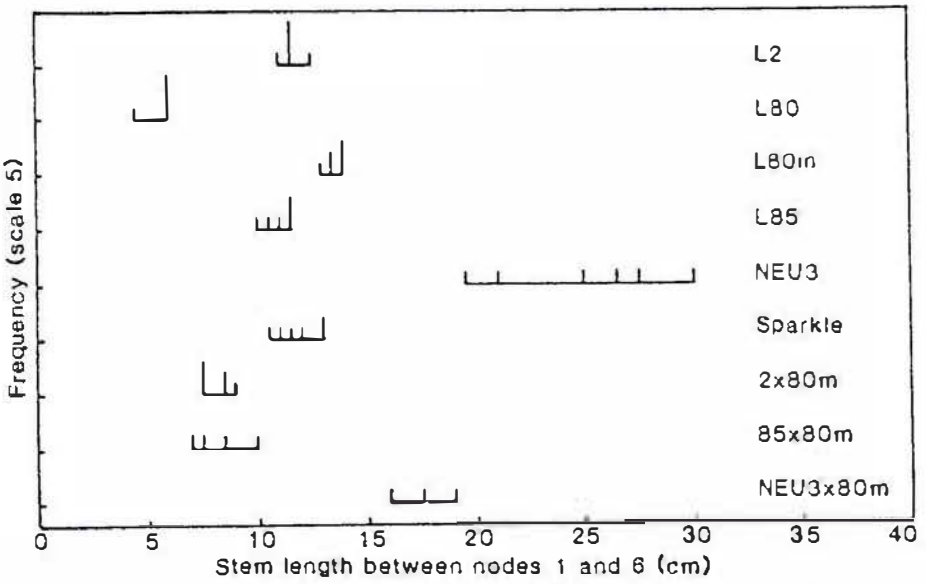

Fig. 2. Distribution of stem length between nodes 1 and 6 for dwarf lines L2, L80, L85 and Sparkle, elongated mutant lines L80m and NEU3, and F_1 plants of crosses 2 x 80m, 85 x 80m and NEU3 x 80m. Conditions: continuous white fluorescent light (6 µmol m^{-2} s^{-1}) at 17.5°C.

indicate monogenic recessive inheritance of the mutant type. Elongated segregates bred true in subsequent generations. The mutant-type segregates were also readily distinguished under this fluorescent light regime by reason of the epinasty of the leaf petioles, and the failure of the leaflets to expand normally. The data in Fig. 2 indicate mutant L80m is allelic with *lv* since the F_1 of cross L80m x NEU3 (*lv*) was also an elongated type with a length intermediate between that of the two parents. The F_1 plants of crosses L80m (*le La Cry* lv^{80m}) x L2 (*le la Cry Lv*), and L80m x L85 (*le La* cry^c *Lv*) were dwarf and somewhat shorter than either parent, presumably because the F_1 plants possess all three dominant alleles *La*, *Cry* and *Lv* (see 3).

The F_2 data from cross Wt3527 (dwarf initial line) x Wt10895 (elongated mutant) likewise indicate monogenic recessive inheritance of the mutant type (Fig. 3). The observed numbers of 43 dwarf and 10 elongated segregates are in good accordance with a 3:1 ratio ($\chi^2 = 1.06$, $P > 0.3$). The elongated types bred true in subsequent generations. The data from the cross Wt10895 x NEU3 (*lv*) indicate that mutant Wt10895 is also allelic with *lv*, since the F_1 plants were elongated types intermediate in length between the two parents (Fig. 4). In contrast, the F_1 of crosses NEU3 x Wt3527 and Wt10895 x Sparkle were dwarf in phenotype and no greater in length than cv. Sparkle (Fig. 4).

Four elongated mutants have now been identified as resulting from mutation of gene *Lv* (8; Figs 2 and 4). The relative strengths of the four alleles lv^{NEU3} (type line NEU3), lv^{R83} (type line R83), lv^{80m} (type line L80m) and lv^{10895}(type line Wt10895) remain to be determined.

Acknowledgements. We thank Dr T.A. LaRue and Professor Dr W.K. Swiecicki, and the late Professors R. Lamm and G.A. Marx, for providing material, Peter Bobbi and Katherine McPherson for technical assistance, and the Australian Research Council for financial support.

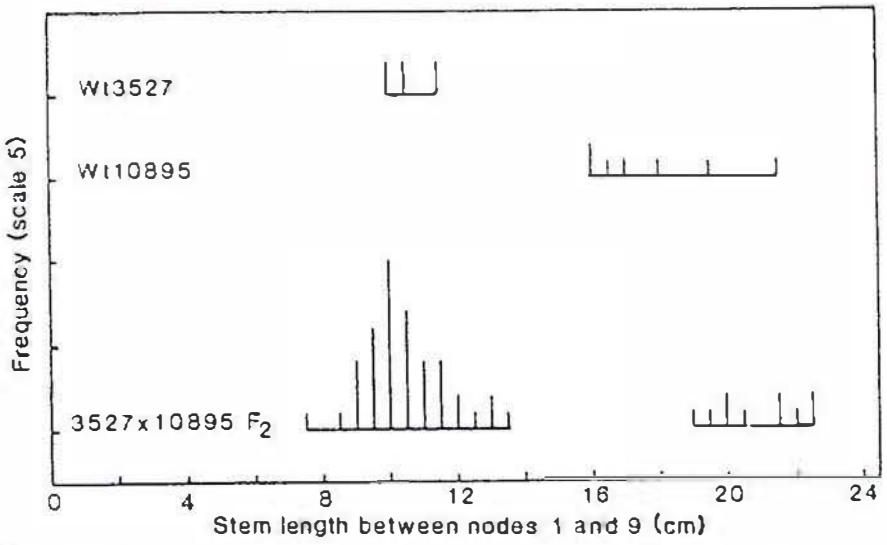

Fig. 3. Distribution of stem length between nodes 1 and 9 for the elongated mutant line Wt10895, the initial line Wt3527 (cv. Paloma), and the F_2 of cross 3527 x 10895. Conditions: photoperiod 8 h (8 h daylight at about 18-22°C and 16 h dark at 16°C).

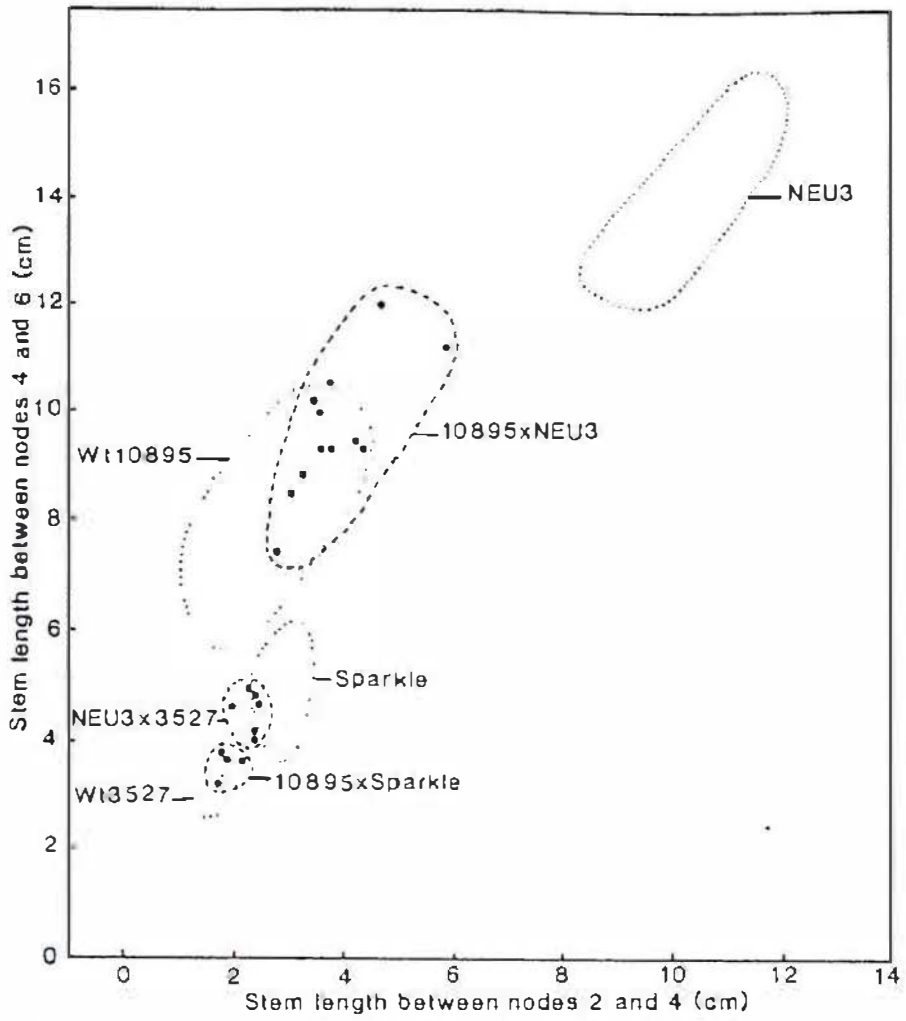

Fig. 4. Stem length between nodes 4 and 6 plotted against stem length between nodes 2 and 4 for mutant line NEU3 and its initial line Sparkle, mutant line Wt10895 and its initial line Wt3527, and the F_1 (●) of crosses 10895 x NEU3, NEU3 x 3527 and 10895 x Sparkle. The plots for the parental lines fell within the respective dotted boundaries (n = 6 except for Wt10895 where n = 18). Conditions: continuous white fluorescent light (90 μmol m^{-2} s^{-1}) at 20°C.

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Photoperiodism and photocontrol of stem elongation in two photomorphogenic mutants of *Pisum sativum* L.

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Abstract. The photomorphogenic mutation *lv* in the garden pea (*Pisum sativum* L.), which appears to reduce the response to light-stable phytochrome, has been isolated on a tall, late photoperiodic genetic background and its effects further characterised. Plants possessing *lv* have a ...ced flowering response to photoperiod relative to wild-type plants, indicating that light-stable phytochrome may have a flower-inhibitory role in the flowering response of long-day plants to photoperiod. In general, *lv* plants are longer and have reduced leaf development relative to *Lv* plants. These differences are maximised under continuous light from fluorescent lamps (containing negligible far-red (FR) light), and decrease with addition of FR to the incident light. Enrichment of white light ... fluorescent lamps with FR promotes stem elonga... in the wild type but causes a reduction in elongation in the *lv* mutant. This "negative" shade-avoidance response appears to be the consequence of a strong inhibitory effect of light rich in FR, revealed in *lv* plants in the absence of a normal response to red (R) light. These results indicate that the wild-type response to the R:FR ratio may be comprised of two distinct photoresponses, one in which FR supplementation promotes elongation ...educing the inhibitory effect of R, and the other in ...h light rich in FR actively inhibits elongation. This ...ypothesis is discussed in relation to functional differentiation of phytochrome types in the light-grown plant. Gene *lw* has been reported previously to reduce internode length and the response to gibberellin A_1, and to delay flowering. The present study shows that the *lw* mutation confers an increased response to photoperiod. In all these responses the *lw* phenotype is superficially "opposite" to the *lv* phenotype. The possibility that the ...ation might primarily affect light perception was therefore considered. The degree of dwarfing of *lw* plants was found to depend upon light quality and quantity. Dwarfing is more extreme in plants grown under continuous R light than in those grown in continuous FR or blue light or in darkness. Studies of the fluence-rate response show that the *lw* mutation imparts a lower fluence requirement for inhibition of elongation by white light from fluorescent lamps. Dark-grown *lw* plants are more strongly inhibited by a R pulse than are wild-type plants but, as in the wild type, this inhibition remains reversible by FR. Light-grown *lw* plants show an exaggerated elongation response to end-of-day FR light. Taken together, these findings indicate that the *lw* mutant may be hypersensitive to phytochrome action.

Key words: Elongation (stem) – Light (red: far-red ratio, end-of-day far-red) – Mutant (photomorphogenic) – Photoperiodism (flowering) – Phytochrome (response) – *Pisum* (mutant, photoperiodism)

Introduction

The red/far-red (R/FR) reversible chromoprotein phytochrome is by far the best characterised of the photoreceptor pigments involved in plant photomorphogenesis (for reviews, see Furuya 1989; Smith and Whitelam 1990; Tomizawa et al. 1990; Quail 1991). While early hypotheses concerning the method of phytochrome action assumed that a single homogeneous pool of phytochrome was responsible for the control of all R/FR reversible phenomena in plant development, it is now well established that there exist at least three distinct molecular species of phytochrome, which differ in photoregulation (Konomi et al. 1987; Sharrock and Quail 1989) as well as in primary structure (Dehesh et al. 1991; Sharrock and Quail 1989) and immunological reactivity (Abe et al. 1985; Somers et al. 1991; Wang et al. 1991). Physiological studies have also indicated that light control of some developmental processes may in-

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Abbreviations: B = blue light; cv. = cultivar; EOD-FR = end-of-day far-red light; FR = far-red light; GA = gibberellin; P_{fr} = far-red-light-absorbing form of phytochrome; phyA, phyB = phytochromes A, B; R = red light; W = white light; WT = wild type

volve at least two pools of phytochrome (the "labile" and "stable" pools), distinguished by differences in the persistence of FR reversibility (Furuya 1989; Smith and Whitelam 1990; Tomizawa et al. 1990). However, direct evidence identifying the physiologically labile and stable pools with different molecular forms of phytochrome is still lacking.

In recent years, several mutants showing reduced response to phytochrome have been identified and characterised (Adamse et al. 1987; Chory et al. 1989; Koornneef et al. 1980, 1985; Nagatani et al. 1990), and have provided some of the strongest evidence for the functional differentiation of the different molecular forms of phytochrome. These mutants all show reduced photoinhibition of stem elongation at the seedling stage, but differ in the complement of phytochrome responses which are altered. Some, such as the *au* mutant in tomato (Koornneef et al. 1985) seem to be deficient in a subset of phytochrome-controlled responses which are associated with the de-etiolation process. Etiolated seedlings lack phytochrome control of elongation and show reduced induction of *cab* synthesis. However, light-grown plants still show some normal phytochrome responses (e.g. end-of-day far-red [EOD-FR] response; Adamse et al. 1988b; Lopez-Juez et al. 1990b). Molecular analysis has shown the *au* mutant to be severely deficient in phytochrome A (phyA; Koornneef et al. 1985; Parks et al. 1987; Quail 1991). In contrast, other mutants such as *lh* in cucumber (Adamse et al. 1987) and *hy3* in *Arabidopsis* (Koornneef et al. 1980) show reduced response to EOD-FR (Lopez-Juez et al. 1990a; Nagatani et al. 1991a) and are deficient in several other phytochrome responses normally present in light-grown plants (Adamse et al. 1987, 1988a; Goto et al. 1991; Whitelam and Smith 1991). The *lh* mutant retains the R/FR reversible low-fluence response for inhibition of hypocotyl elongation in etiolated seedlings (Adamse et al. 1987). Both of these mutants have recently been shown to lack the light-stable phytochrome B (phyB; Lopez-Juez et al. 1992; Nagatani et al. 1991a; Somers et al. 1991), thus strongly implicating phyB in control of the EOD-FR response.

Recent work has also focused on mutants or transgenic plants which possess elevated levels of phytochrome or which show increased response to phytochrome action. One such mutant is the phytochrome-hypersensitive high-pigment (*hp*) mutant of tomato (Peters et al. 1989). Mature white-light (W)-grown *hp* plants are dwarfed and have darker green foliage than do wild-type (WT) plants. The mutation confers increased sensitivity both to inhibition of hypocotyl elongation by red (R) and blue (B) light, and to induction of anthocyanin synthesis by R. Overexpression of monocotyledonous *phyA* genes in transgenic tobacco (Boylan and Quail 1989; Keller et al. 1989) and *Arabidopsis* (Boylan and Quail 1991) also results in dwarfism and increased leaf and fruit pigmentation, as well as decreased apical dominance. Physiologically, constitutive phyA production results in persistence of an etiolated-seedling high-irradiance response (HIR) in de-etiolated plants, altered photosensitivity of inhibition of stem elongation, and disruption of the "shade-avoidance" response to low R:FR ratio (McCormac et al. 1991; Nagatani et al. 1991b). Overexpression of *phyB* genes in transgenic *Arabidopsis* (Wagner et al. 1991) has also been shown to confer a light-dependent, short-hypocotyl phenotype. Thus it is clear, both from these results and from the results obtained from phytochrome-deficient mutants, that both labile phytochrome (phyA) and a stable phytochrome (phyB) have a role in the photoinhibition of stem elongation.

The recent discovery of multiple distinct phytochrome-encoding genes (Sharrock and Quail 1989; Sommers et al. 1991), and the possibility that the proteins encoded by these genes may have partially overlapping and/or co-operative function (Kay et al. 1989; McCormac et al. 1991; Wagner et al. 1991) have increased our appreciation of the complexity of phytochrome-mediated photoregulation of development and required re-evaluation of long-standing assumptions. Nonetheless, it is obvious that further characterization of existing photomorphogenic mutants and the identification of others will be of considerable value in dissecting out the roles of these different phytochrome types.

We have previously described a range of mutations affecting stem elongation in pea (Jolly et al. 1987; Reid et al. 1983, 1991, 1992; Reid and Potts 1986; Reid and Ross 1988, 1989). These mutants have been primarily examined with respect to gibberellin (GA) involvement, and some have been shown directly to affect GA synthesis or catabolism (mutants *le, lh, ls, na* and *sln*). Others alter the response of the plant to biologically active GA (e.g. *la, cry*^{s}, *lk, lka, lkb, lkc*). Our investigation of the possibility that altered elongation may result from aberrant light perception has so far resulted in the characterization of the "GA-hypersensitive" mutant *lv* (Reid and Ross 1988) as a photomorphogenic mutant (Nagatani et al. 1990). This mutant shows a reduction in R inhibition of stem elongation, lack of an EOD-FR elongation response and reduced chlorophyll levels. The similarity of the *lv* mutant to the phyB-deficient mutants *lh* in cucumber and *hy3* in *Arabidopsis*, particularly with respect to the lack of an EOD-FR response, indicates that the *lv* mutation may specifically reduce the response to phyB. Although it has previously been suggested that *lv* may block transduction of the signal from light-stable phytochrome (Nagatani et al. 1990), the effect of the mutation on phytochrome levels will require further examination in light of the recent identification of phytochromes D and E (Somers et al. 1991).

As two major roles of phytochrome in the green plant are in photoperiodism and the detection of light quality (Smith and Whitelam 1990), and the nature of phytochrome action in the green plant is still far from clear, it is of considerable interest to examine the effect of stable-phytochrome altered mutants on these processes. In this study we have partially characterized the effects of the *lv* mutation on photoperiodism and the so-called shade avoidance response to low R:FR ratio, and have further characterized the effect of the mutation on the response to EOD-FR.

In contrast to *lv*, the dwarf pea mutant *lw* has reduced response to applied GA (Jolly et al. 1987). Furthermore,

lw plants develop severe water congestion and show a substantial delay in flowering (Jolly et al. 1987). These pleiotropic effects of the *lw* mutation indicate that the gene has an important developmental role. In this paper, we also report on investigation of the flowering response to photoperiod and the photocontrol of stem elongation in *lw*.

Materials and methods

Plant material. The pure lines of pea used in this work are held in the collection at Hobart, Australia. All lines used are dominant at the internode length loci *Le, Na, Lh, Ls, Lk, Lka, Lkb* and *Lkc* and are dominant at at least one of the duplicate loci *La* and *Cry*, unless otherwise specified. The origin and initial characterization of the dwarf mutant K29 (*lw*) is detailed in Jolly et al. (1987). Line 232− extremely tall, photoperiodic line carrying the photomorphogenic mutation *lv* and the flowering genes *Sn, Dne* and *hr* (see Murfet (1985) for review of flowering genes in pea). Lines 232− and 232+ (*Lv*) are essentially isogenic lines derived from the same plant in the F_6 of cross cultivar (cv.) Torsdag (WT) × NEU3(*le cry^s lv*; Reid and Ross 1988; Reid 1989). The day-neutral (*sn*) background of the original *lv* line NEU3 (Reid and Ross 1988) has hitherto precluded examination of the effect of *lv* on photoperiodism.

Growing conditions. All plants were grown in a 50:50 mixture of vermiculite and dolerite chips topped with 20–30 mm of potting mix in either 140-mm-diameter slimline pots at two plants per pot (R reversibility, E●D-FR and photoperiod experiments) or in 400 × 300 × 100 mm^3 toteboxes in a 6 × 8 array (light quality and fluence experiments). Plants used in the photoperiod experiment received an 8-h photoperiod of natural daylight in a heated glasshouse maintained at 23° C, and were transferred into night compartments at 16° C where the photoperiod was extended with incandescent light from Thorn 40-W globes at a photon fluence rate of ≈3 μmol · m^{-2} · s^{-1}. These plants were supplied weekly with Aquasol from two weeks after sowing, and lateral shoots were regularly excised. All other plants were grown in controlled-environment cabinets held at 20° C.

White light was supplied from fluorescent tubes (40 W, Thorn cool-white), supplemented where required with incandescent globes (Thorn 60 W). Red, B and FR sources were identical to those used by Nagatani et al. (1990). Dark-grown plants were measured under a dim green safelight. In all experiments, only main shoots were scored, and nodes were counted from the cotyledonary node as zero. Plant age was determined from the day of sowing, and at 20° C shoots emerged from the growth medium 4–5 d after sowing.

Results

Photoperiod reponse of lv and lw mutants. In order to examine the response of *lv* to photoperiod, we compared the flowering and elongation of lines 232+ and 232− in 8-, 12-, 16- and 24-h photoperiods. K29 (*lw*) and its progenitor cv. Torsdag (also flowering genotype *Sn Dne hr*) were also included in this experiment, with the intention of further characterizing the effect of *lw* on flowering reported by Jolly et al. (1987).

There was no significant difference in the node of flower initiation (NFI) of line 232− and 232+ in the 24-h photoperiod, with both lines flowering at about node 14. However, in the 8-h photoperiod, line 232− flowered significantly earlier (NFI = 15.6) than line 232+ (NFI = 20.7; Fig. 1A). These results indicate a substantial reduction in the flowering response to photoperiod of line 232−, which flowered only 1.5 nodes later ($P < 0.001$) in 8 h than in 24 h (compared with 6 nodes in line 232+). On the basis of NFI, it appears that *lv* imparts a marked reduction in the ability to respond to photoperiod. This reduction in response to the photoperiod extension is also reflected in measurements of internode length (Table 1). However, the abortion of flower initials in the 8-h photoperiod (Fig. 1A, broken line) and the retention of a small effect of the photoperiod extension on both NFI (Fig. 1A) and inter-

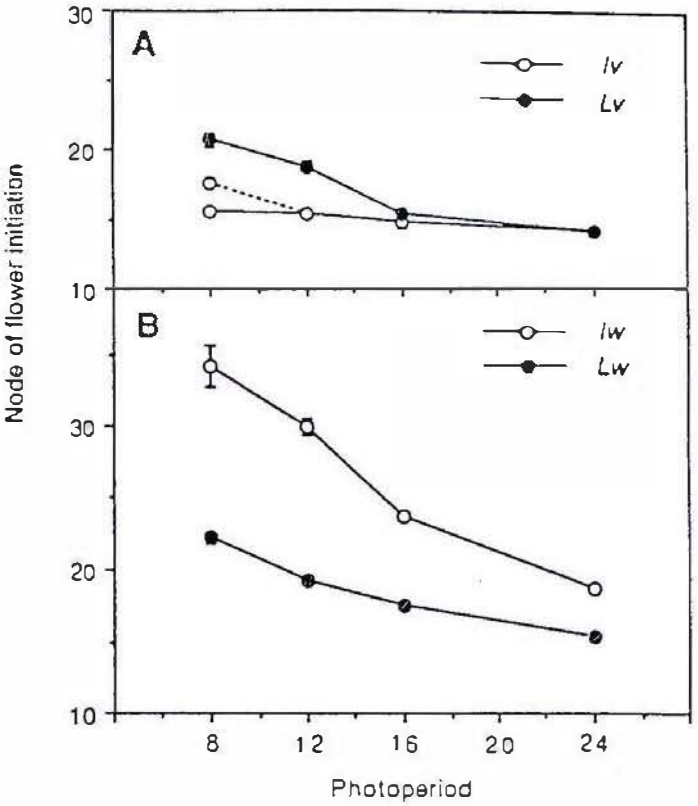

Fig. 1A, B. Node of first initiated flower ± SE for pea lines 232+ (*Lv*) and 232− (*lv*) (A), and Torsdag (*Lw*) and K29 (*lw*) (B), grown in 8-, 12-, 16-, and 24-h photoperiods. Each datum point represents the mean from 8–12 plants. The broken line in A indicates the mean node of first developed flower for line 232−. In no other instance did the node of flower development differ from the node of flower initiation

Table 1. The mean stem length in cm ± SE between nodes 1 and 9 (L_{1-9}) of line 232+ (*Lv*), 232− (*lv*), cv. Torsdag (*Lw*) and K29 (*lw*) pea plants grown in a photoperiod of 8 or 24 h. The response index (RI) for each line was calculated as the ratio of L_{1-9} in the 24-h and 8-h photoperiods. $n = 8$–12

Photoperiod	Genotype			
	Lv	*lv*	*Lw*	*lw*
24 h	65.1 ±1.2	79.2 ±3.2	51.5 ±0.9	33.0 ±0.5
8 h	34.3 ±0.9	60.7 ±2.1	27.1 ±0.7	16.5 ±0.3
RI	1.89	1.30	1.90	2.00

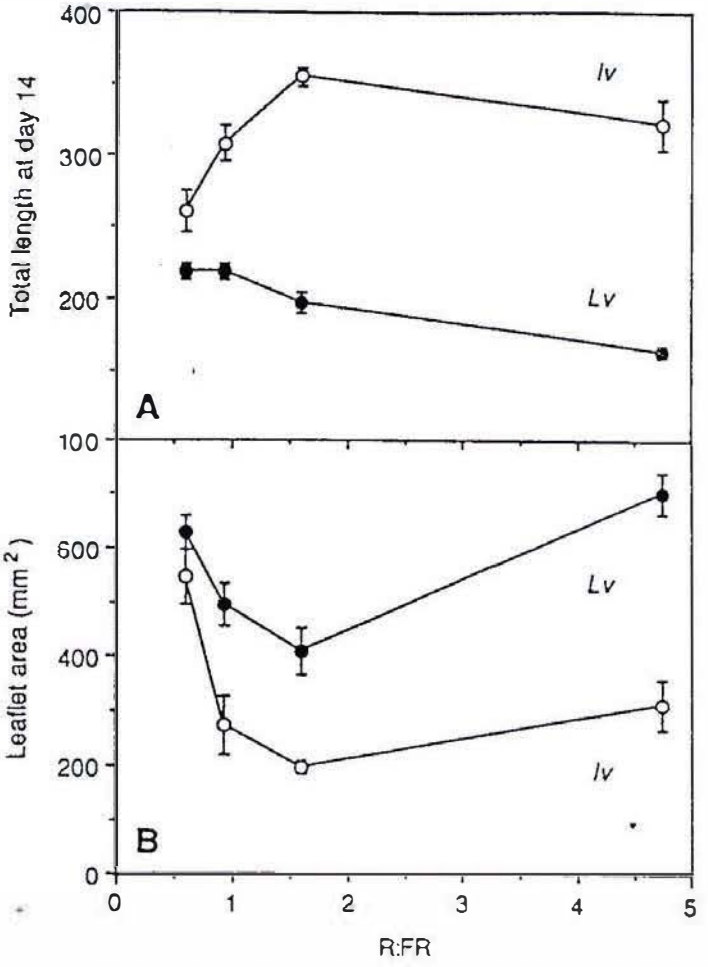

Fig. 2A, B. Responses of pea plant lines 232+ (*Lv*) and 232− (*lv*) to variation in R:FR ratio. A Total shoot length of 14-day-old plants (±SE), measured from soil surface to tip of apical bud. B Area of largest leaflet at node 4 (mm^2) estimated as maximum length × maximum width. ±SE. All plants were grown in white light providing photosynthetically active radiation of 60 µmol · $m^{-2} \cdot s^{-1}$ (measured as total photon fluence between 400 and 700 nm). The ratio R:FR was varied by supplementation of white light from fluorescent tubes (R:FR = 4.8) with that from incandescent globes. R:FR was measured as the ratio of fluence rates within the 650- to 660-nm and 720- to 730-nm wavebands. $n = 10$–12

node length (Table 1) indicate that *lv* plants retain some sensitivity to photoperiod. The perfect coincidence of the altered flowering and internode-length traits in *lv* segregates, and observations that another *lv* allele of independent origin confers a qualitatively similar reduction in sensitivity to photoperiod (data not shown) effectively rule out the possibility that the altered flowering behaviour of line 232− is a consequence of a closely linked gene or an effect of the genetic background.

K29 showed an increased response to photoperiod, with a delay in NFI of 15 nodes across the photoperiod range compared with a 6-node delay in cv. Torsdag (Fig. 1B), although the difference between K29 and the WT was minimised in the 24-h photoperiod, flowering in K29 was still delayed by three nodes in these conditions. Even though cv. Torsdag showed a substantial elongation response to the daylength extension, the *lv* mutation caused a small increase in this response, indicated by the response indices in Table 1.

Table 2. Shoot length in cm (measured from soil surface to tip of apical bud) ± SE of 14-d-old pea plants of lines 232+ (*Lv*), 232− (*lv*), cv. Torsdag (*Lv*) and K29 (*lv*) grown in an 8-h photoperiod under white light (160 µmol · $m^{-2} \cdot s^{-1}$ at pot top) from fluorescent lamps and either transferred directly to darkness following the daily photoperiod (− FR) or given a 20-min exposure to FR before transfer to darkness (+ FR). The response index (RI) was calculated as the ratio of the mean shoot lengths of FR-treated and untreated plants (+ FR, − FR). $n = 10$–12

Treatment	Genotype			
	Lv	*lv*	*Lv*	*lv*
− FR	19.3 ±0.5	29.5 ±0.5	19.3 ±0.4	9.8 ±0.2
+ FR	26.0 ±0.5	28.3 ±0.3	28.1 ±0.7	18.7 ±0.2
RI	1.35	0.96	1.46	1.91

Response of the lv mutant to R:FR ratio. The capacity of plants to perceive and respond to the ratio of R to FR in the incident light is well known (Smith 1982), and it has been speculated that perception of the R:FR ratio may be attributable to stable phytochrome (phytochrome-S: Smith et al. 1991). We compared the response of lines 232 − (*lv*) and 232 + (*Lv*) to changing R:FR ratio. Line 232+ showed a 34% decrease in stem elongation in response to increasing R:FR from a minimum of 0.6 to a maximum of 4.8 (Fig. 2A). Elongation of line 232+ decreased in a roughly linear manner with decreased FR content of the incident light, with the lowest FR content (i.e. highest R:FR) eliciting the greatest inhibition of elongation. Line 232− was longer than line 232+ under all R:FR ratios used but, in contrast to line 232+, maximum inhibition of elongation in line 232− occurred in light with the highest FR content (i.e. lowest R:FR). The 19% overall increase in elongation from lowest to highest R:FR in line 232− was thus in marked contrast to the decrease shown by line 232+, indicating that high R:FR was much less effective at inhibiting elongation in line 232− than in line 232+ and that FR actively inhibited elongation in *lv* plants.

Leaflet area in line 232+ was increased by both high and low R:FR ratios, with intermediate ratios being less effective (Fig. 2B). Although line 232− exhibited a response for leaf development which was similarly shaped to that shown by line 232+, leaf area was reduced in line 232− relative to line 232+ at all R:FR ratios. The difference in leaf development between the two genotypes was maximised at high R:FR, due to an apparent marked reduction in the ability of line 232− to respond to high R:FR.

The EOD-FR response of lv and lw mutants. After 14 d growth, line-232+ plants given a 20-min FR treatment at the end of an 8-h photoperiod were 35% longer than plants not receiving the EOD-FR treatment (Table 2). In contrast, EOD-FR treatment caused no significant elongation of line-232− (*lv*) plants; rather, a small (4%) but significant ($P < 0.05$) reduction in total length was observed. These results are borne out in the response of individual internodes (Fig. 3A).

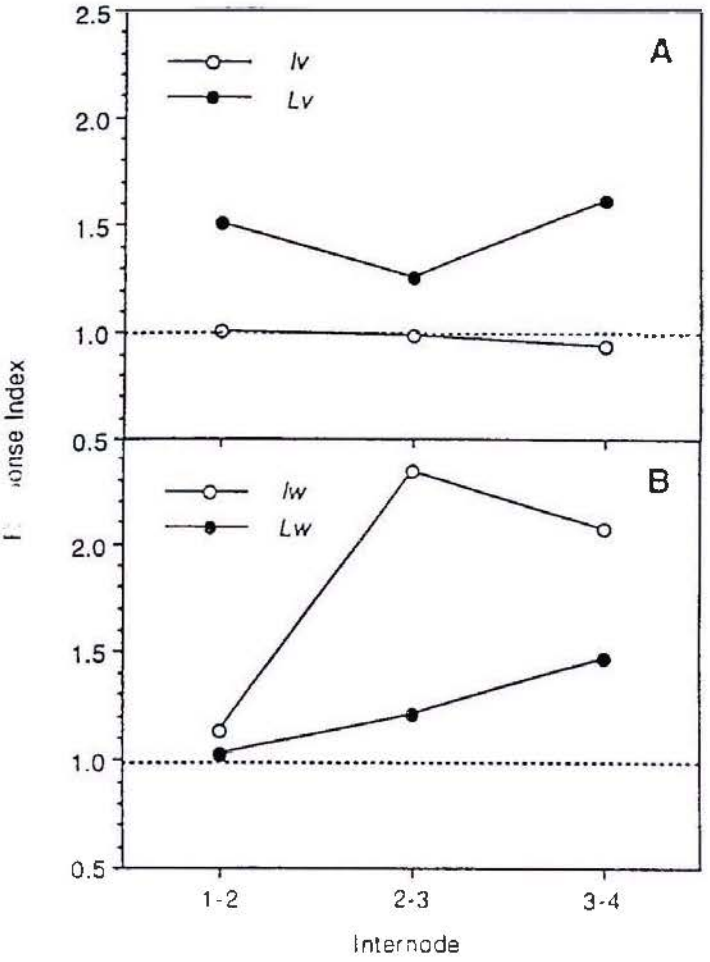

Fig. 3A, B. Indices of response for the elongation response to EOD-FR of individual pea internodes in lines 232+ (*Lv*) and 232− (*lv*), (A), and cv. Torsdag (*Lw*) and K29 (*lw*) (B). The response index was calculated as the ratio of the internode lengths of EOD-FR-treated and untreated plants. $n = 10–12$

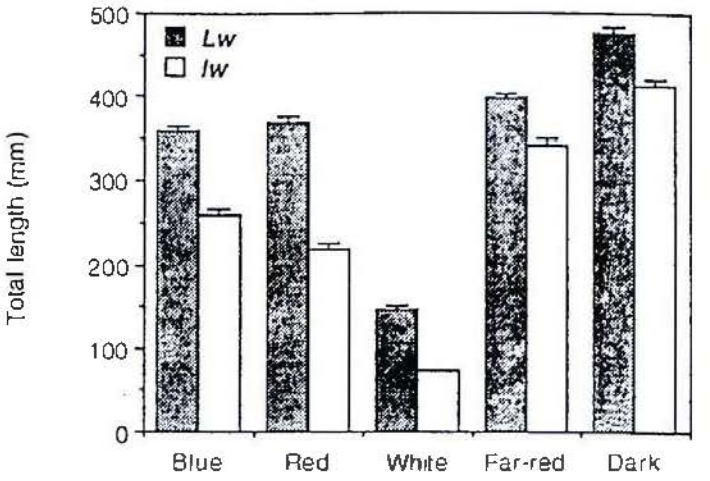

Fig. 4. Total shoot length (measured from soil surface to tip of apical bud) ± SE for 14-d-old pea plants of cv. Torsdag (*Lw*) and mutant K29 (*lw*) grown under either continuous white (90 μmol · m^{-2} · s^{-1}), red (6 μmol · m^{-2} · s^{-1}), blue (6 μmol · m^{-2} · s^{-1}) or far-red light (4·5 μmol · m^{-2} · s^{-1}) or in darkness. $n = 10–12$

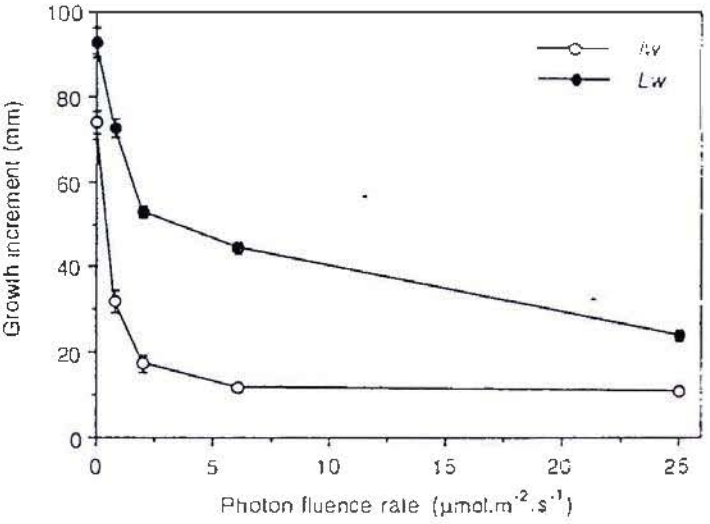

Fig. 5. Mean increase in shoot length ± SE of etiolated seedlings of pea cv. Torsdag (*Lw*) and mutant K29 (*lw*) over the 48 h following transfer (7 d after sowing) from darkness to continuous "fluorescent" W. $n = 10–12$

We also examined the EOD-FR response of K29. Whereas EOD-FR treatment resulted in a 46% increase in elongation in the WT (Table 2), the relative effect of this treatment on K29 was much greater (91%). This alteration of the EOD-FR response of K29 was seen most prominently in the second internode (Fig. 3B) which showed a 140% increase in the mutant compared with a 20% increase in the WT.

Response of the lw mutant to light quality. The growth of *lw* and *Lw* plants was compared under broad-band R, FR and B, "fluorescent" W, and in the dark (Fig. 4). When grown in the dark, *lw* plants were only 13% shorter than WT plants. A similar reduction in length was seen in K29 plants grown in FR, indicating that FR had little effect on the expression of the mutation. However, the *lw* difference was expressed to a much greater extent in R (41% reduction) and in W (51% reduction). The effect of B was intermediate, causing a 27% reduction in elongation of the mutant relative to the WT. These results indicate that the expression of *lw* varies with light quality. It is interesting to observe that the order of effectiveness of these light treatments (W ≥ R > B > FR) corresponds with the order of effectiveness of transformation of phytochrome to the FR-absorbing (P_{fr}) form.

Fluence-rate response of stem elongation in the lw mutant. The *Lw*/*lw* elongation difference in "fluorescent" W was examined across a range of fluence rates. The results in Fig. 5 clearly show that expression of the mutation is dependent on irradiance. The dwarfing effect of the *lw* mutation is most clearly expressed in relatively low-fluence light (73% reduction in length relative to WT at 6 μmol · m^{-2} · s^{-1}) and is expressed to a lesser extent at higher irradiances (54% reduction at 25 μmol · m^{-2} · s^{-1}) and in darkness (20% reduction). The mutation thus appears to impart an increased sensitivity to dim W. In a separate experiment employing similar growth conditions, K29 seedlings grown under 90 μmol · m^{-2} · s^{-1}

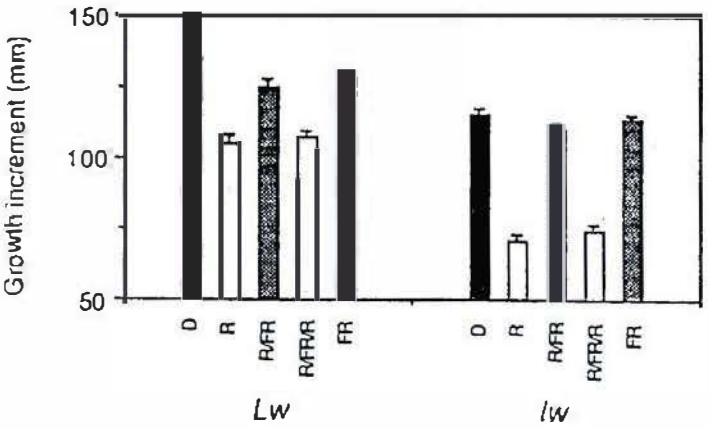

Fig. 6. The mean increase in shoot length + SE for pea plants, cv. Torsdag (*Lw*) and K29 (*lw*), exposed to red light (*R*), far-red light (*FR*), R followed by FR (*R/FR*), R followed by FR followed by R (*R/FR/R*), or continuous darkness (*D*). Irradiation with each light type lasted for 20 min and was given to 8-d-old etiolated seedlings (shoots 60–80 mm long). Plants were measured at the time of irradiation and again after 66 h. $n = 10$–12

"fluorescent" W showed only a 25% reduction in length relative to cv. Torsdag, providing a further indication that expression of the mutation is reduced at high W irradiances.

Red/far-red reversibility of elongation in the lw mutant. A 20-min irradiation of 8-d-old *Lw* seedlings with R (30 μmol $\cdot$ m^2 $\cdot$ s^{-1}) resulted in a significant 22% inhibition of stem elongation over the subsequent 66-h period (Fig. 6) This inhibition was partially reversed by 20 min FR given directly after the R treatment. Far-red light alone had no significant effect on stem elongation ($P > 0.1$). Plants possessing *lw* showed a qualitatively similar response, but the extent of the R-induced inhibition was much greater than in *Lw* plants, both in relative (38% versus 22%) and in absolute (44 mm versus 30 mm) terms. This result strongly indicates that the increased response of the *lw* mutant to dim W reflects a general-sense hypersensitivity to phytochrome action.

Discussion

Previous characterization of the *lv* mutant (Nagatani et al. 1990) has shown it to be phenotypically similar to the phyB-deficient mutants *lh* in cucumber and *hy3* in *Arabidopsis*, in that all three mutants show reduced inhibition of elongation by R and lack an elongation response to EOD-FR (Adamse et al. 1987; Lopez-Juez et al. 1990a, 1992; Nagatani et al. 1991a). In this study, we have demonstrated a further phenotypic similarity between the *lv* and *hy3* mutants, namely, a substantial reduction in flowering response to photoperiod relative to WT plants (Fig. 1A; Goto et al. 1991). However, like *hy3*, *lv* does not completely eliminate the photoperiod response. This is evident in the small delay in flower initiation (in short days compared with long days; Fig. 1A), the slightly larger delay in flower development (Fig. 1A), and by the increase in stem elongation (Table I) in *lv* plants. This additional similarity of *lv* to *hy3* adds support to the suggestion that *lv* may reduce the response to the phyB signal. If so, it also lends support to the conclusion of Goto et al. (1991) that reduced response to phyB diminishes the delaying effect of short days on flower initiation in long day plants (LDPs).

It seems that phyB can therefore be considered to have an inhibitory or delaying function in photoperiodic flower induction in LDPs. However, such a function does not conveniently fit the existing concept of phytochrome action in LDP photoperiodism, which views the promoting effect of long days as being mediated by phytochrome in the P_{fr} form. Even where FR is more effective in promoting flowering than R (e.g. when given as a photoperiod extension; Reid and Murfet 1977; Carr-Smith et al. 1989) the response has often been interpreted as a form of high-irradiance response (HIR) and generally presumed to act through P_{fr}. While our results do not rule out the possibility that P_{fr} has a flower-promotory function, they do provide strong evidence for a distinct, inhibitory function of phytochrome in photoperiodic flower induction. Such a function is in keeping with the observations of several workers (e.g. Schneider et al. 1967; Holland and Vince 1971; Evans 1976) that reduction in the ratio of Pfr to total phytochrome (P_{fr}/P_{tot}) at an appropriate time in the daily cycle promotes flowering in LDP. Along these lines, Vince-Prue (1986) has argued that the FR action maxima observed by many workers for promotion of flowering in LDP by long exposures to light are not explicable solely in terms of P_{fr}. In addition, physiological studies of photoperiodism in the short day plants *Lemna paucicostata* and *Pharbitis nil* have also revealed both inhibitory and promotory effects of FR, attributed to a labile and stable phytochrome, respectively (Takimoto and Saji 1984; Lumsden et al. 1987), and it is conceivable that a similar situation may exist in LDPs. If so, the residual response to photoperiod seen in *lv* may represent that component of the total photoperiod response mediated by the phytochrome unaffected by the mutation.

Response of the lv mutant to R:FR ratio. The elongation response of light-grown plants to low R:FR ratio has been reported to show a linear relationship with the proportion of total phytochrome in the P_{fr} form (Smith 1982). The relative simplicity of this relationship has been taken to reflect the involvement of only one type of phytochrome. The response of WT pea plants is in keeping with this view when R:FR ratios (Fig. 2A) are expressed as phytochrome equilibrium values (P_{fr}/P_{tot}). Mutants deficient in or with reduced response to phytochrome might therefore be expected to show a reduced elongation response to low R:FR, as well as an overall increase in length. This has been found to be the case for photomorphogenic mutants *hyl*, *hy2*, *hy3* and *hy6* in *Arabidopsis*, *au* in tomato and *lh* in cucumber (Whitelam and Smith 1991), although none of these mutants completely lack the response. In *lv* pea plants, however, low R:FR actually causes a *reduction* in elongation relative to high R:FR (Fig. 2A). Such a reduction has only ever

previously been reported in phyA-overexpressing transgenic tobacco (McCormac et al. 1991). These findings are difficult to reconcile with the current understanding of phytochrome control of the shade-avoidance response. However, viewing the etiolated condition as the "ground state" more appropriately emphasises the action (or lack of action) of phytochrome in light-induced inhibition of stem elongation and allows a clearer understanding of the behaviour of *lv*. Viewed in this way, light rich in FR (low R:FR ratio) is almost as effective at inhibition of elongation in *lv* as in *Lv* (Fig. 2A), whereas light at high R:FR is much less effective than low R:FR light at inhibition of elongation in *lv*, despite being more effective in *Lv*. The inhibition of elongation in *Lv* at high R:FR obviously indicates a strong inhibitory response to R, which can be reduced by increasing the proportion of FR in incident light, but is still relatively strong across the R:FR range used. The dramatic reduction in inhibition of *lv* plants at high R:FR indicates that they lack this strong inhibitory response to R, and it appears that in the absence of this response, a second inhibitory response to light rich in FR is revealed. Similarly, the leaf-development response of *Lv* (Fig. 2B) may be understood in terms of promotory responses to high R:FR and to low R:FR, and the response of *lv* understood if the mutant lacks the response to high R:FR but retains the response to R:FR.

The EOD-FR response of the lv mutant. Plants possessing *lv* have previously been reported to lack an elongation response to EOD-FR (Nagatani et al. 1990). While the results of the present study confirm this observation, they also reveal a small but significant decrease in elongation in response to EOD-FR (Table 2, Fig. 3). A similar small decrease in elongation in response to EOD-FR treatment was also reported for the *lh* mutant of cucumber (Lopez-Juez et al. 1990a), although this was dismissed as a response to plant handling. However, in view of the clear inhibition of elongation in *lv* at low R:FR (Fig. 2A) we suggest that the small decrease in length in EOD-FR-treated *lv* and *lh* plants may reflect a real inhibitory effect of FR (i.e. the second responsediscussed above) revealed in the absence of a normal R-inhibitory response to phyB. It seems likely that the inhibitory effects of low R:FR and EOD-FR seen in *lv* may have the same physiological basis, with the difference in magnitude attributable to the difference in duration of exposure to light rich in FR.

Thus the results from photoperiod, EOD-FR and R:FR experiments taken together can be interpreted to indicate that *lv* is deficient in response to a phytochrome species which is normally inhibitory to flowering, inhibitory to stem elongation, and promotory to leaf development. In the EOD-FR and R:FR experiments this was manifest as a reduced response to R. However, in each instance *lv* plants retained some sensitivity to the varying light conditions employed. While the residual response to photoperiod may be interpreted in terms of "leakiness" of the mutation, and hence in terms of control through a single phytochrome response, the residual responses to EOD-FR and R:FR are opposite in direction to the response of the WT and therefore more difficult to interpret in this way. We feel that these results may indicate two physiologically distinct responses to phytochrome, one of which is markedly reduced in *lv* plants. With respect to both inhibition of elongation and promotion of leaf development, we envisage a state in the light-grown plant in which two photoresponses are operative, one in which R is the most effective and which depends on R:FR (equivalent to the "shade-avoidance response"), and the second in which FR alone is effective (possibly equivalent to the standard FR-HIR: e.g. Beggs et al. 1980). Detection of the FR response in *lv* and in phyA-overexpressing plants can be explained if the FR response is controlled by phyA but is usually masked by phyB action (Smith and Whitelam 1990). Overexpression of phyA may increase the FR response to a level detectable over a normal phyB response, effectively suppressing the latter (McCormac et al. 1991), while in *lv*, the reduction in response to phyB may allow detection of the normal FR-HIR. It is possible that the response of *lv* to photoperiod may also reflect the involvement of two such photoresponses. Although attractive, such an explanation is obviously largely speculative. In order to demonstrate conclusively the discrete nature of these two putative responses and to relate more closely the behaviour of *lv* to that of *lh* and *hy3*, it will be necessary to determine phyB levels and to undertake more detailed characterisation of the residual phytochrome and photoperiod responses of the mutant.

Photocontrol of elongation in the lw mutant. The *lw* mutation, originally characterized by Jolly et al. (1987), has reduced sensitivity to GA, reduced internode length and delayed flowering relative to its WT. In these three respects, the *lw* phenotype is opposite to the *lv* phenotype. Furthermore, we found in this study that *lw* increased the flowering response to photoperiod (Fig. 1), and the elongation response to EOD-FR (Fig. 2, Table 2), indicating that light perception in the mutant might be altered. Subsequent experiments revealed that the mutation confers a hypersensitivity to inhibition of elongation both by continuous low-fluence W (Fig. 5) and by a short pulse of R (Fig. 6). Expression of the mutant phenotype is dependent on light quality (Fig. 4) and fluence rate (Fig. 5), and *lw* can therefore be considered a photomorphogenic mutant.

The response of *lw* appears similar to the "phytochrome-hypersensitive" mutant *hp* of tomato (Peters et al. 1989). Hypocotyl growth of *hp* was reduced relative to the WT under low-fluence B and R, and the mutant was hypersensitive to induction of anthocyanin synthesis by R. However, a more striking comparison may be made between *lw* and the transgenic tobacco line CR overexpressing rice phyA (Nagatani et al. 1991b). In contrast to a previous report (Kay et al. 1989), the mature transgenic plants showed a distinct morphological phenotype, with shorter stems and darker foliage. An examination of segregation of the transgene (on several genetic backgrounds) showed that expression of the dwarf phenotype was dependent on irradiance. Wild-type and transgenic seedlings could be clearly distin-

guished in dim W but not in darkness or in higher irradiance W. Furthermore, transgenic CR plants showed an increased inhibition of stem elongation in response to brief R pulses, but retained full FR reversibility of this response. The similarity of *lw* to CR plants in these two respects make the speculation that the *lw* mutation is closely associated with phytochrome action particularly attractive and deserving of further investigation.

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Location of the *Lv* gene in pea linkage group VI

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Mutant *lv* plants are characterised by elongated internodes when grown under red or white light but are indistinguishable from wild type *Lv* plants in darkness or under far-red light (4,12). Mutant plants also lack a normal elongation response to end-of-day far-red light and to a low red : far-red ratio (11) and are earlier flowering under short (non-inductive) photoperiods (11). Four recessive *lv* alleles have been identified from the mutant lines NEU3, R83, Wt10895 and L80m (5,10), with each conferring a similar phenotype (10,12). Because expression of the *Lv-lv* difference is restricted to certain light conditions, the *lv* mutants can be termed photomorphogenic. The syndrome of photomorphogenic abnormalities seen in the *lv* mutants is indicative of a reduction in the function of phytochrome B (phyB), one of several related photoreceptor proteins which play a major role in the control of plant development by light. Recent results have shown that lines R83, Wt10895 and L80m are all deficient in the phyB apoprotein while the NEU3 mutant has normal levels of phyB (12) suggesting that *Lv* may be a structural gene for phy B.

We report here data showing that the *lv* locus is in linkage group VI between *wlo* and *Prx3*, and close to *na* (within 2 cM).

F_2 segregation data for *lv* and group VI primary markers *wlo, na, Prx3, Arg* and *Pl* [see mapping guidelines (9)] were obtained from three crosses as detailed in Table 1. Parental marker lines 111 (A875-55-0) and 224 (A783-161) come from the Marx collection, line 107 is a selection from cv Torsdag, and the *lv* allele in line 232 is derived from mutant line NEU3. Further details of the lines used are given in previous papers (10,11).

Identification of the *Lv* - *lv* segregation was facilitated by growing the plants for the first 10 days in a growth chamber at 20°C under continuous white light (150 μmol $m^{-2}s^{-1}$ at pot top) supplied by 40W cool white fluorescent tubes. The plants were then transferred to the glasshouse and grown to maturity under an 18 h photoperiod. All crosses were of normal fertility. Data were analysed using the programs Linkage-1 (6) and CROS (S.M. Rozov).

All individual segregations in Table 1 are in accordance with expectation ($P>0.05$). The joint segregation data reveal strong linkage of *lv* with *na* (<2 cM), *wlo* (4 cM) and *Prx3* (8 cM) and moderate linkage with *Arg* (26 cM) and *Pl* (26 cM) with $P < 0.000001$ and < 0.0001, respectively.

Table 1. F_2 segregation data for *lv* and linkage group VI markers.

Genes		Cross[a]	Phenotype[b]						Total	Chi-squared Locus 1	Chi-squared Locus 2	Chi-squared Joint	Linkage Prob.	Recomb. Fraction	SE
			DD	DR	RD	RR									
Lv	*Wlo*	1	99	2	3	24			128	1.04	1.50	99.41	<0.0001	4.2	1.8
Lv	*Arg*	1	87	14	13	14			128	1.04	0.67	17.99	<0.0001	26.2	4.7
Lv	*Pl*	1	87	14	13	14			128	1.04	0.67	17.99	<0.0001	26.2	4.7
Wlo	*Arg*	1	86	16	14	12			128	1.50	0.67	11.25	<0.001	30.2	5.0
Arg	*Pl*	1	99	1	1	27			128	0.67	0.67	116.56	<0.0001	1.7	1.1
Wlo	*Pl*	1	86	16	14	12			128	1.50	0.67	11.25	<0.001	30.2	5.0
Lv	*Wlo*	2	45	30	24	0			99	0.03	1.48	13.77	<0.001		
Lv	*Na*	3	130	3	2	46			181	0.22	0.41	156.44	<0.0001	2.7	1.2
			DF	DH	DS	RF	RH	RS							
Lv	*Prx3*	2	3	32	25	19	3	0	82	0.15	1.98	54.83	<0.0001	7.6	3.0
Wlo	*Prx3*	2	22	32	6	0	3	19	82	0.15	1.98	44.80	<0.0001	11.0	3.6

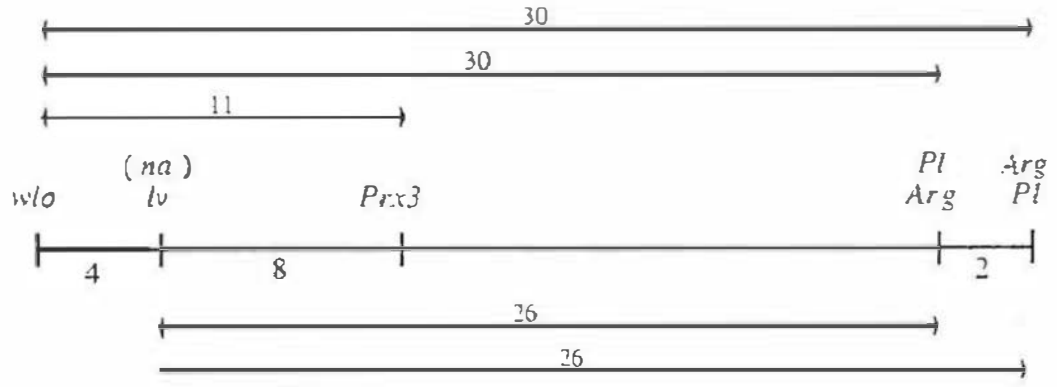
[a]Cross: 1) line 80m (*lv wlo arg pl*) x line 224 (*Lv Wlo Arg Pl*)

2) line 232 (*lv Wlo* $Prx3^F$) x line 111 (*Lv wlo* $Prx3^S$)

3) line 107 (*Lv Na*) x *lv na* segregate from cross NEU3 (*lv Na*) x L81 (*Lv na*)

[b]D = dominant, R = recessive, F = homozygous fast, H = heterozygous, and S = homozygous slow. The first named locus is shown first.

The data in Table 1 generate the following map:



These results place *wlo* substantially closer to *Prx3*, *Arg* and *Pl* than the distance shown in recent maps (7,8). The discrepancy relates specifically to the distance *wlo* to *Prx3* which is about one third of that shown in the latest map (8). The distances for *Prx3* - *Arg*

and *Prx3 - Pl* are consistent with recent maps. Moreover, our data for *wlo - Pl* and *wlo - Arg* are entirely consistent with values obtained from very large data sets by Lamprecht (1) and Marx (2,3). Based on a sample of 2797 plants, Lamprecht reported the *wlo - Pl* distance as 31.6 ± 1.1 cM. Likewise, Marx' data indicate about 30 cM for the *wlo - Arg* distance. In the absence of multi-point data the map position of *na* remains unclear. Our 2-point data place *na* and *lv* in close proximity and imply that *na* may lie between *wlo* and *pl* as shown in Marx'(2) tentative map. However, the majority of Marx' 1981 (2) and 1982 (3) results in fact support the conclusion that *na* lies in the upper section of group VI above *wlo*. Likewise, our data do not indicate whether *Pl* lies between *Arg* and *wlo* as shown by Marx (2,3) or the reverse arrangement as shown on the latest map (8). Our data for the *Arg - Pl* joint segregation are very similar to those of Marx and confirm tight linkage between these two loci.

In summary, these results obtained from three different crosses and using two different *lv* alleles are consistent and they provide convincing evidence that *lv* is located in linkage group **VI** between *wlo* and *Prx3* and close to *na*. The distance between loci in the upper and lower sections of this linkage group may be less than shown in the current map (8). We have planned a 5-point coupling phase cross involving standard line JI1794 and markers *wlo, lv, Gty, Prx3* and *Pl* to further examine distances in this section of group VI and other crosses to determine the position of *na*.

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Gibberellins and phytochrome regulation of stem elongation in pea

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Abstract. In garden pea (*Pisum sativum* L.) neither etiolation nor the phytochrome B (phyB)-response mutation *lv* substantially alters the level of the major active endogenous gibberellin, GA_1 in the apical portion of young seedlings. The phyB-controlled responses to continuous red light and end-of-day far-red light are retained even in a GA-overproducing mutant (*sln*). Comparison of the effects of the *lv* mutation and GA_1 application on seedling development shows important differences in rate of node development, cell extension and division, and leaf development. These results suggest that in pea the control of stem elongation by light in general and phyB in particular is not mediated by changes in GA_1 content. Instead, the increased elongation of dark-grown and *lv* plants appears to result from increased responsiveness of the plant to its endogenous levels of GA_1. Three GA_1-deficient mutants, *na*, *ls* and *le* have been used to investigate these changes in responsiveness, and study of these and the double mutants *na lv*, *ls lv* and *le lv* has demonstrated that the relative magnitude of the change in responsiveness is dependent on GA_1 level. The difference in pleiotropic effects of GA_1 application and the *lv* mutation suggest that light and GA_1 interact late in their respective transduction pathways. A model for the relationship between light, GA_1 level and elongation in pea is presented and discussed.

Key words: Gibberellin (levels, response) – Light (phytochrome B) – Mutant (photomorphogenic, gibberellin) – *Pisum* (mutants) – Stem elongation

Introduction

Many studies have addressed the hypothesis that gibberellins (GAs) may mediate the effects of light on stem elongation. These studies have examined the effects of light on the levels and metabolism of various GAs, and on the response to applied GAs. While the involvement of changes in GA levels with photoperiod-induced bolting of rosette plants is well established (e.g. Talon and Zeevaart, 1990), effects of the gross white light (W) or red light (R) dark difference or end-of-day far-red (E●D–FR) treatment on the levels of active GAs have been less conclusively demonstrated.

Recent studies which have used gas chromatography-selected ion monitoring (GC–SIM) for GA quantification have reported such changes in relation both to the light dark difference (Toyomasu et al. 1992) and to R and far-red light (FR) treatment (Fang et al. 1991), and have concluded that the effects of these various light regimes on elongation are at least partly mediated by changes in the level of active GAs. Similar conclusions have been reached from GA application and metabolism studies (e.g. Campell and Bonner 1986; Martinez-Garcia and Garcia-Martinez 1992b). Other recent evidence has come from the photomorphogenic mutants *ein* in *Brassica* and ma_3^R in *Sorghum*. These mutants show a dramatic reduction in the inhibition of elongation by R, are deficient in a light-stable phytochrome species (Childs et al. 1991; Devlin et al. 1992) tentatively identified as phytochrome B (phy B) and are both reported to have elevated levels of GA_1 (Rood et al. 1990a, b; Beall et al. 1991). In both cases it has been suggested that the increase in GA_1 levels is a direct consequence of the reduced phytochrome response (Childs et al. 1991; Devlin et al. 1992). These results raise anew the question of whether responses to phyB, such as the high-irradiance response to R (Parks and Quail 1993) and the related EOD-FR and "shade-avoidance" responses (Nagatani et al. 1991; Whitelam and Smith 1991), involve changes in GA levels.

However, in our work with pea and sweet pea, we have accumulated considerable evidence that light does not act by altering the level of the major active endogenous GA, GA_1, but rather, by altering the responsiveness of the plant to GA_1. We have found this to be true for the gross light/dark comparison (Reid 1988,

Abbreviations: B, blue light; cv., cultivar; E●D-FR, end-of-day far-red light; FR, far-red light; GA_n, Gibberellin A_n; GC-SIM, gas chromatography-selected ion monitoring; HIR, high irradiance response; W, white light

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Ross and Reid 1989; Ross et al. 1992b) as well as in the case of elongation in response to a FR-rich photoperiod extension (Reid et al. 1990). These results are in direct contrast to the frequently cited conclusion of Campell and Bonner (1986), who suggested on the basis of GA-application studies that phytochrome might regulate the conversion of GA_{20} to GA_1 in pea and hence, presumably, the level of GA_1. We have also shown that the pea photomorphogenic mutant *lv*, which is deficient in response to phyB (Nagatani et al. 1990; Weller and Reid 1993) is hyper-responsive to applied GA_1 (Reid and Ross 1988), while preliminary analysis of GA content in *lv* (by bioassay) indicated normal levels of GA-like substances in this mutant (Reid and Ross, 1988).

In view of recent reports of the effects of light and phyB on GA levels, we have examined both the GA levels in the phyB-response mutant *lv*, and light responses in the mutant *sln* which, at the seedling stage, possesses very high levels of GA_1 (Reid et al. 1992; Ross et al. 1993a). We have included *sln* in our quantifications as an additional control to indicate the effects of elevated GA_1 level. Use of the *lv* and *sln* mutants has thus allowed us to study two major aspects of the *ein* and ma_3^R phenotype (reduction in phyB response and elevated levels of active GAs) separately, and to investigate their relationship. We conclude that the response to phyB in pea is not mediated by changes in GA_1 level, but instead, suggest that light perceived by phytochrome acts in part to alter the responsiveness of the plant to its endogenous GA_1.

Materials and methods

Plant material. Hobart pea (*Pisum sativum* L.) lines 232$^+$ (*Lv*) and 232$^-$ (*lv*) are essentially isogenic lines derived from a common F_6 ancestor (Weller and Reid 1993). The *sln* and *Sln* plants used in this study derive from homozygous *sln* and *Sln* families in the B_3 generation of the backcross [NGB6074 (*sln*) × cv. Torsdag (*Sln*) F_1] × NGB6074 (Reid et al. 1992), except in the case of the light-quality experiment (Fig. 4) where NGB6074 and the cultivar, Torsdag were used. The *na* and *na lv* plants (Figs. 6, 7) were F_5 progeny of homozygous segregates from the same plant in the F_3 of the cross Hobart line 81 (*le na*; Reid et al. 1983) × NEU3 (*le Lv*; Reid and Ross 1988), whereas the *ls* and *ls lv* plants (Figs. 6, 7) were a random selection of segregates in the F_3 of the cross Hobart line 181 (*Le ls*; Reid 1988) × L232$^-$ (*Le lv*). The origin of the line NEU3 (*le lv*) from the cultivar Sparkle (*le Lv*) has been described previously (Reid and Ross 1988).

Growing conditions. Plants were grown in pots containing a 1 : 1 (v/v) mixture of vermiculite and dolerite chips, topped with 2–3 cm of potting soil. All plants were grown in controlled-environment cabinets at 20 °C. All W-grown plants were grown under light from cool-white fluorescent tubes providing a photon flux density of 150 μmol · m^{-2} · s^{-1}, with the exception of plants in the EOD–FR experiment which received a photon flux density of 60 μmol · m^{-2} · s^{-1}. The blue light (B), R and FR sources for the EOD–FR and light-quality experiments were identical to those used by Nagatani et al. (1990) and Weller and Reid (1993). Nodes were counted from the cotyledonary node as zero, and plant age was determined from the day of sowing. Where required, GA_1 was applied to the exposed cotyledon of dry seeds in 5 μl ethanol using a micropipette, prior to sowing. Control plants were treated with ethanol only.

Quantification of GAs. Details of plants harvested are contained in Table 1. The harvested apical portion of light-grown plants comprised that part of the plant above the uppermost expanded leaf (leaf 4 in experiment 1, leaf 4 or 5 in experiment 2). This resulted in roughly the same proportion of stem harvested for each genotype (approx. 18% of total length at day of harvest). However, the different morphologies and rates of leaf expansion in light- and dark-grown plants in experiment 1 rendered difficult the choice of a comparable sample from dark-grown plants. We decided to base our comparison upon proportion of total stem length, and harvested the uppermost 5.5 cm of dark-grown plants (approx. 18% of total length). The harvested leaf portion (experiment 2) comprised the uppermost expanded leaf including tendrils, petiole and stipules. The internode portion comprised the full length of the uppermost fully expanded internode, and the greater fresh weight of this portion from *Lv* compared with *Lv* plants reflected the twofold greater length of this internode in *lv* plants.

Harvested tissue was immersed in cold (−20 °C) methanol. The harvested material was homogenised, and internal standards added. For both experiments, the internal standards were [17,17-$^2H_2$]GA_{19}, [17,17-$^2H_2$]GA_{20}, [17,17-$^2H_2$]GA_1, [17,17-$^2H_2$]GA_{29}, [17,17-$^2H_2$]GA_8 and [17,17-$^2H_2$]GA_{29}-catabolite, provided by Professor L. Mander (Australian National University, Canberra, A.C.T.). The amounts of each internal standard added to the extracts are shown in Table 1. Extraction of GAs was performed as described elsewhere (Reid et al., 1990). The GA-containing fractions from HPLC were dried, derivatised and analysed using the GC–SIM system as described previously (Reid et al. 1990; Ross et al. 1993a). Samples containing certain GAs (particularly GA_{19}) were analysed using an HP5890 series II gas chromatograph coupled via direct inlet to a Kratos Concept ISQ mass spectrometer operating (in the SIM mode) at a resolution of 10000. For these samples a BP1 GC column (SGE, Melbourne, Australia) was employed, using a temperature program which ran from 60 to 240 °C at 30 °C · min^{-1} and thereafter to 276 °C at 3 °C · min^{-1}, and a pressure rising from 25 to 35 psi at 1 psi · min^{-1}, with helium as the carrier gas.

Chlorophyll quantification. Single leaflets from leaf 4 of 14-d-old seedlings were extracted in dimethylformamide for 24 h in darkness at 4 °C. Chlorophyll content was determined from the absorbance of the extracts at 647 and 664.5 nm using the equations of Inskeep and Bloom (1985).

Results

Comparison of GA levels in light- and dark-grown plants. Under the conditions employed, dark-grown wild-type pea plants were approximately 3 times longer than light-grown plants both in terms of total length (Table 1) and internode length (Table 2). However, the GA_1 level in the apical portion did not differ substantially between light- and dark-grown plants (Fig. 1). The apical GA_1 level in both cases was well within the range of 6–15 ng · (g FW)$^{-1}$ reported for seedlings of light-grown wild-type pea lines (e.g. Reid et al. 1990, 1992). By way of comparison, light-grown *sln* plants were only about 2 times longer than light-grown wild-type plants (Table 1), but had a GA_1 level of 53 ng · g FW^{-1}, more than 5 times the level in the wild type (9.7 ng · (g FW)$^{-1}$). These results suggest that the inhibitory effect of light on elongation is unlikely to involve any reduction in the level of active GA_1 in expanding tissues.

However, light was not without effect on the levels of other GAs. The GA_{20} concentration in dark-grown plants was reduced to only 3% of the level in light-grown plants (Fig. 1), a dramatic decrease in keeping with previous observations from pea (Ross and Reid 1989) and sweet pea (Ross et al. 1992). Both GA_{29} and GA_{29}-catabolite were also decreased in dark-grown plants,

Table 1. Harvest details of pea plants grown for quantification of gibberellin levels in light- and dark-grown wild-type seedlings, and in light-grown *lv* seedlings

Expt.	Environment	Genotype	Height from soil to tip of plant (cm)	Age at harvest (d)	No. expanded leaves	No. plants	Plant portion	FW of harvested tissue (g)	Amount of internal standard added (ng)					
									GA_{19}	GA_{20}	GA_1	GA_{29}	GA_8	GA_{29}-cat
1	Light	*Sln*	9.5±0.2	10	4–5	49	apex	8.32	25	250	70	70	130	500
		sln	22.0±0.5	10	4–5	45	apex	10.88	55	1000	500	1200	1000	800
	Dark	*Sln*	30.1±0.5	10	3	48	apex	14.06	40	40	110	40	225	840
2	Light	*Lv*	17.1±0.6	15	4–5	60	internode	8.58	50	100	35	30	90	130
							apex	22.70	60	400	230	300	750	340
							leaf	18.48	30	550	115	500	700	240
		lv	34.0±0.8	15	4–5	57	internode	20.82	60	45	115	50	300	400
							apex	19.09	50	320	190	270	570	250
							leaf	18.45	50	570	160	500	670	240

Table 2. Comparison of the effects of GA_1 application and the *lv* mutation in 14-d-old pea seedlings. Asterisks indicate those characteristics differentially affected by GA_1 and *lv*. The estimate of leaflet area is calculated as the product of the length and width of a leaflet from leaf 4. $n=10–12$ for the first five characteristics, otherwise $n=6$. Plants were WL-grown except where indicated

Character		Wild-type control	Wild-type + 10 μg GA_1	*lv*
Length of internode 2–3 (cm)		4.2±0.2	10.4±0.3	9.6±0.7
	Dark	17.6±0.7	25.3±0.8	17.9±1.0*
Total length (cm)		18.5±0.4	37.2±2.0	35.6±1.0
	Dark	45.0±0.4	49.2±2.0	44.2±0.5*
Leaves expanded		5.54±0.07	6.08±0.08	5.21±0.05*
Leaflet area (cm^2)		8.94±0.41	5.35±0.35	4.34±0.53
Stem thickness at internode 2–3 (mm)		2.41±0.03	1.85±0.04	2.42±0.08*
Seedling DW (mg)		151±7	160±6	154±5
Seedling FW:DW ratio		10.6±0.2	13.6±0.1	10.7±0.2*
DW leaf:stem ratio		2.64±0.08	0.92±0.04	1.36±0.03
Internode 2–3 cell length (μm)	Epidermis	330±8	692±19	623±11
	Cortex	70±2	94±5	139±3*
Internode 2–3 cell number (μm)	Epidermis	114±4	156±5	140±4*
	Cortex	538±23	1163±62	628±24*
Leaflet chlorophyll content (mg (g FW)$^{-1}$)		3.01±0.05	3.11±0.02	3.17±0.11
Chlorophyll a:b ratio		3.01±0.04	2.68±0.03	2.62±0.02

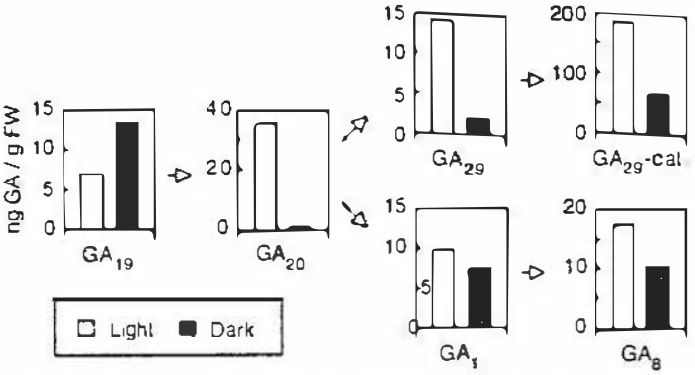

Fig. 1. Gibberellin levels in the apical portion of light- and dark-grown wild-type pea plants. The *arrows* linking the histograms indicate the main pathways for GA metabolism in pea shoots

though not to the same extent as GA_{20}. The level of GA_{19} in dark-grown plants was elevated to approximately twice the wild-type level (see later).

Effect of the lv mutation on GA levels. The *lv* mutation also appears to have little effect on the level of GA_1 in the apical portion from light-grown plants (Fig. 2) despite a twofold increase in length relative to *Lv* plants (Tables 1, 2). The uppermost expanded internode and leaf of *lv* plants contained substantially less GA_1 than the comparable portions from wild-type plants (20% and 40% of wild-type levels, respectively). In addition to GA_1, elevated levels of GA_3 have also been reported for the phyB mutant *ein* in *Brassica* (Rood et al. 1990a, b). However in pea shoots, GA_3 is either absent or present at very low levels, and in another experiment (results not shown) GA_3 was not detected in dark- or light-grown wild-type or *lv* plants.

As in etiolated plants, GA_{20} levels were markedly reduced in *lv* plants; to less than 25% of wild-type levels in all three harvested portions. The levels of GA_{19} were lower in the internode and leaf, while in the leaf and apical portions, GA_8 levels were increased in *lv* plants. The high level of GA_{29}-catabolite in all portions from both genotypes (and in dark-grown plants; Fig. 2) is likely to reflect carryover from the seed, where levels of this metabolite are extremely high (Sponsel 1983; Ross et al. 1993a), and are thus unlikely to relate to GA metabolism in the seedling itself.

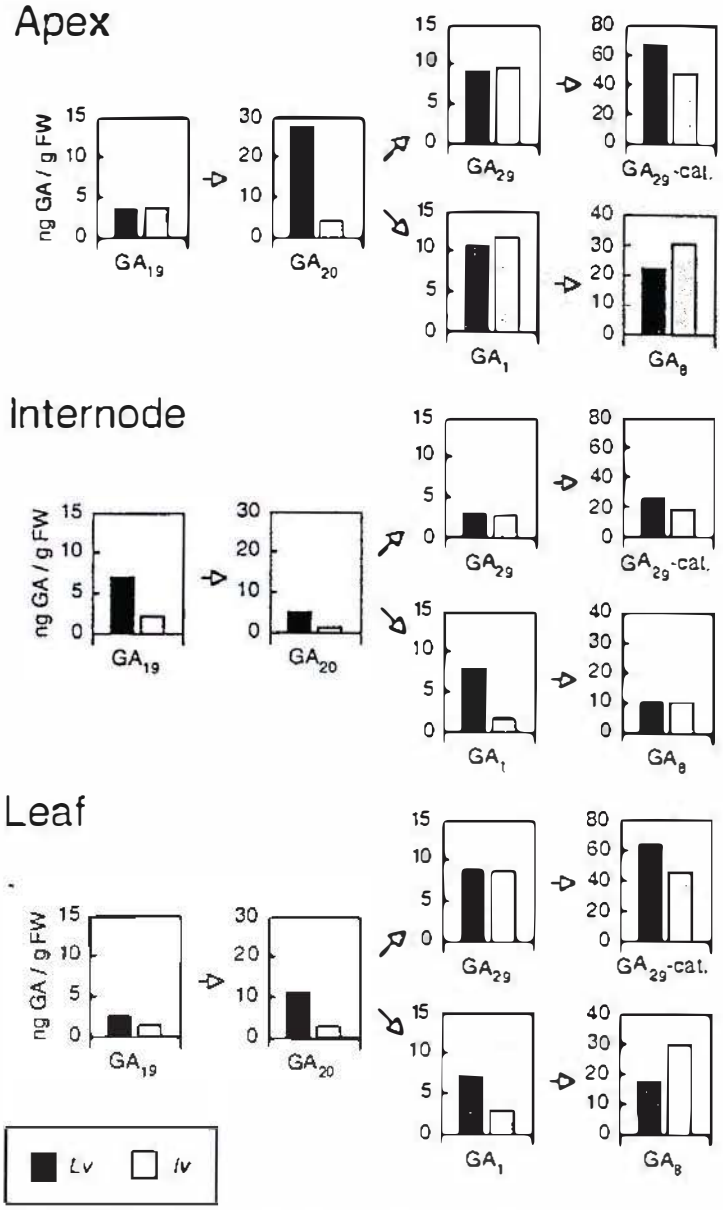

Fig. 2. Gibberellin levels in the apical portion, the uppermost fully expanded internode, and the uppermost fully expanded leaf of light-grown wild-type and *lv* pea seedlings. The *arrows* linking the histograms indicate the main pathways for GA metabolism in pea shoots

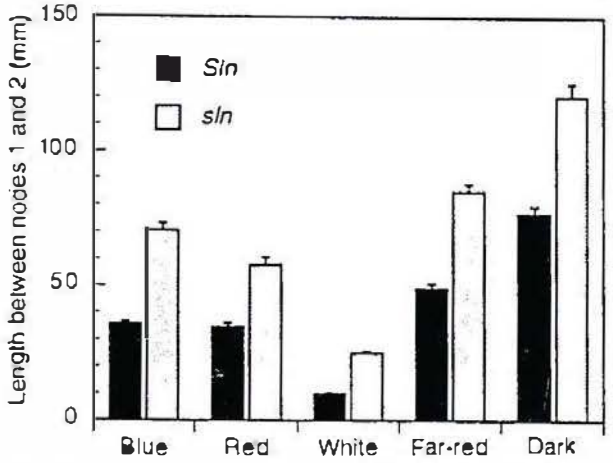

Fig. 3. Length between nodes 1 and 2 (mm) ± SE for plants of the pea cultivar Torsdag (*Sln*) or NGB6074 (*sln*) grown under continuous W, R, B or FR or in complete darkness. $n = 10$–12

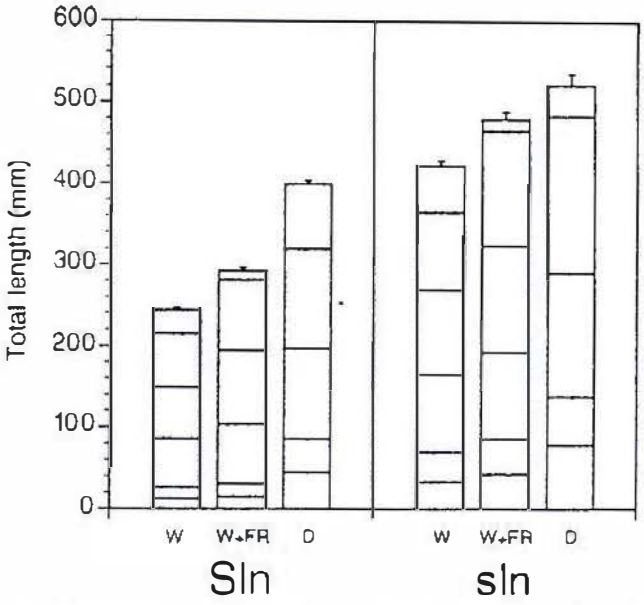

Fig. 4. Total shoot length (mm) ± SE for wild-type *Sln* and GA_1-overproducing mutant *sln* pea plants grown under an 8-h W photoperiod with (*W + FR*) or without (*W*) a 20-min FR extension, or in complete darkness (*D*). The relative positions of nodes are indicated as horizontal lines. $n = 10$–12

Variation in GA levels between organs. The analysis of leaves and internodes in experiment 2 (Fig. 2) gives an indication of the difference in GA content of these organs. In agreement with Smith et al. (1992), GA_{20} levels in wild-type plants were elevated in the leaf relative to the internode. However, the lower level of GA_{19} in the leaf contrasts with the results of Smith et al. (1992) but is in agreement with those of Ross et al. (1993b) from sweet pea. The higher levels of GA_{20} and GA_{29} in leaves relative to stems (Fig. 2) may explain in part the reduction in GA_{20} and GA_{29} levels in dark-grown plants (Fig. 1), where the proportion of leaf to stem tissue in the apical portion is considerably lower than in plants grown in the light. A similar explanation may account for the increase in GA_{19} level in dark-grown plants (Fig. 1).

Light responses of sln plants. We reasoned that if responses to light and to phyB in particular are mediated by changes in GA_1 level, then such responses should not occur in the presence of saturating GA_1 levels. We therefore examined light responses in the GA_1-overproducing mutant *sln* which appears to be effectively saturated for GA_1-induced elongation (Reid et al. 1992; Ross et al. 1993a). Elongation in *sln* plants was reduced in the light relative to darkness (Figs. 3, 4). Responses to continuous B, R and FR were normal (Fig. 3), although *sln* plants were longer than the wild type under all conditions. The mutant also retained a relatively normal, albeit slightly reduced, elongation response to EOD–FR (Fig. 4), with respect to both internode length (increase in L2–3 of 12% cf. 20% in *Sln* plants) and total length at 14 d (14% cf. 21%).

Morphological comparison of GA treatment and the lv mutation. Reports describing mutants *ein* in *Brassica*, ma_3^R in *Sorghum* and *lh* in cucumber have suggested in

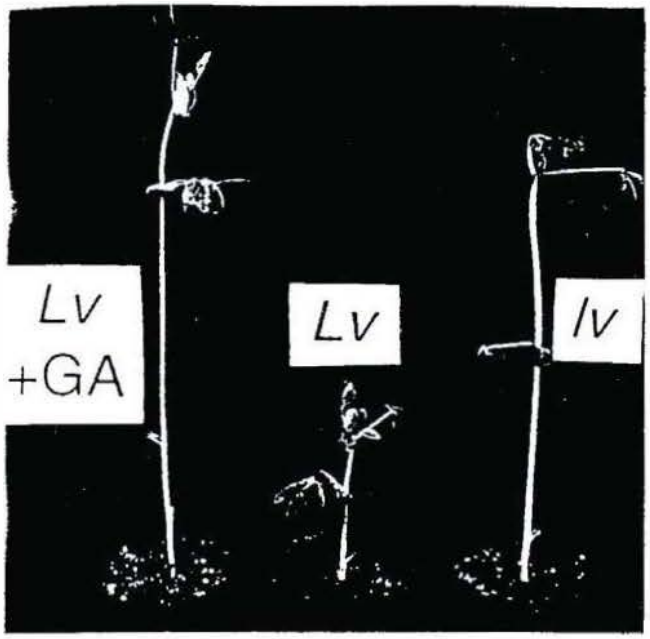

Fig. 5. Comparison of the effects of GA_1 application and the *lv* mutation on the morphology of light-grown wild-type tall pea plants (line 232$^+$ background). The plant on the *left* (genotype *Lv*) was treated on the seed with 10 μg GA_1

each case that the phyB-deficient phenotypes of these mutants can (to a varying extent) be mimicked by application of active GA to the wild type (Lopez-Juez et al. 1990, 1993; Rood et al. 1990a, b; Beall et al. 1991.) These claims could be seen to constitute indirect evidence that the mutant phenotypes result from increased levels of active GA, although alteration to an early step in the GA-signal transduction could conceivably have the same effect. We therefore compared the effects of the *lv* mutation and GA_1 application. After 14 d growth, wild-type plants treated on the seed with 10 μg GA_1 and untreated *lv* plants were indeed very similar in length (Fig. 5), some two- to threefold longer than untreated wild-type plants. Both the *lv* mutation and GA_1 application also had similar effects on other characteristics such as leaflet area and dry-matter allocation to the leaves, as might be expected for elongated phenotypes (Table 2). However, the effects of GA_1 treatment and of the *lv* mutation differed in several major respects (Fig. 5, Table 2). Gibberellin A_1 increased the rate of node development (leaf expansion) relative to untreated plants, while *lv* plants showed retarded node development (Table 2). Leaflets of *lv* plants showed marked curling, failure of the laminae to expand, and pronounced epinasty (Fig. 5). The *lv* mutation had no effect on stem thickness, whereas the stems of GA_1-treated plants were considerably thinner than the control (Table 2). Furthermore, the elongating effects of GA_1 application and of the *lv* mutation could be distinguished at a cellular level where, for cortical tissue, the increase in length of internode 2–3 was due mainly (75%) to an increase in cell length, while the GA_1 treatment acted mainly through an increase in cell number (82%), consistent with cellular effects of light and GA on elongation previously reported for pea (Reid 1983; Reid et al. 1983) and cowpea (Martínez-García and García-Martínez 1992a).

Fig. 6. Comparison of the effects of genetically increasing endogenous GA_1 content in light-grown *Lv* plants, light-grown *lv* plants and dark-grown *Lv* plants (from left). In each treatment group the four plants have genetic background *na*, *ls*, *le* and wild type (respectively, from left). All plants are 14 d old

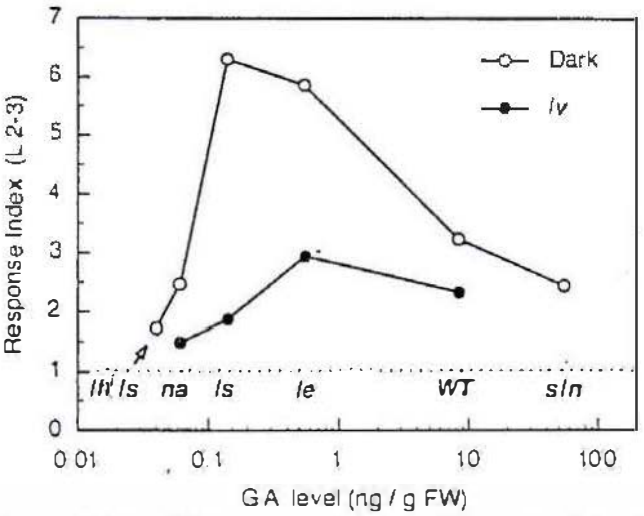

Fig. 7. Relationship between endogenous GA_1 level and the elongation response of internode 2–3 to darkness and the *lv* mutation. The light-response data are derived from mutants *na*, *ls*, *le*, *sln*, the double mutant *ls lh* and wild-type (*WT*) plants grown in W or in complete darkness, and mutant *lv* and double mutants *na lv*, *ls lv* and *le lv* grown in the light. Response indices are calculated as (length in darkness/length in light) or (length of *lv* double mutant/single mutant). The GA_1 level for the *sln* mutant was obtained from results in the present study, while all other GA_1 levels are derived from Swain (1993). These values are approximate only, being based in some cases on quantification from mutants on different genetic backgrounds from those used here (see *Materials and methods*). They are also based on the assumption that neither darkness nor *lv* substantially alter GA_1 levels on any of the backgrounds shown (see Figs. 2 and 3)

Interaction between lv and GA-deficient mutants. We further examined the relationship between endogenous GA_1 level and the response to light using GA-deficient mutants in double-mutant combinations with *lv*. Figure 6 illustrates the effect of etiolation and the *lv* mutation on four genetic backgrounds (*na*, *ls*, *le* and wild type) which span a range in endogenous GA_1 content of more than two orders of magnitude; from approximately 0.06 ng · (g FW)$^{-1}$ in *na* plants to around 10 ng · g^{-1} in the wild type (Swain, 1993). The difference in elongation between etiolated and light-grown plants, or between *Lv*

and *lv* plants is dependent on GA_1 level, being reduced at the extremes of GA_1 concentration (Fig. 7) where GA_1 levels are either so low that virtually no elongation can occur, or are approaching saturation.

Discussion

Quantification of GA_1 from the apical portion of dark and light-grown wild-type pea seedlings has revealed no substantial difference in the level of GA_1, despite a threefold difference in length (Fig. 1, Table 1). This result has been confirmed in replicate experiments, supports previous bioassay results (Ross and Reid 1989), and is in agreement with similar quantifications from sweet pea (Ross et al. 1992). These results argue strongly against the hypothesis that the increased length of dark-grown plants relative to W- or R-grown plants is due, at least in part, to an increase in the level of GA_1 (as suggested by Campell and Bonner 1986).

Similarly, the GA_1 level in the apical portion of *lv* plants is not substantially elevated relative to the wild type, despite a twofold increase in length (Fig. 2, Table 1). Since the *lv* mutant is very similar in phenotype to known phyB-deficient mutants *hy3* in *Arabidopsis* and *lh* in cucumber (Lopez-Juez et al. 1990; Nagatani et al. 1990, 1991; Weller and Reid 1993), this result suggests that responses to phyB such as inhibition of elongation by continuous R (R–HIR), and elongation in response to EOD–FR treatment are not mediated through changes in the level of active GA in expanding tissue. This is in contrast to results from the *ein* and ma_3^R mutants, which both show a greatly reduced R–HIR, but are reported to have elevated levels of active GAs (Childs et al. 1991; Devlin et al. 1992).

It could be suggested that the failure to detect an increase in the level of active GAs in dark-grown wild-type or light-grown *lv* plants might result from "dilution" effects in the larger tissue portions harvested from these plants. We feel that such an effect is unlikely to have obscured any substantial difference in GA_1 content, given that in all cases the harvested apical portion consisted entirely of expanding tissue, in which GA_1 is still active in the promotion of elongation. Hence, if the increased elongation in these plants was due to increased GA_1 levels, we would still expect to find a substantial increase in GA_1 in the apical portion as harvested. It might also be argued that the failure to find a light-induced change in GA_1 level does not entirely rule out the involvement of active GAs in responses to light. For example, light might direct the compartmentalization of GAs in the apical portion so as to alter the availability of GA_1 for the promotion of elongation, without altering the overall GA_1 concentration. This may be true, and more detailed investigation of localization of GA_1 within the apical portion is necessary. However, much of the precedent for the suggested involvement of GAs in the response to light has come from reports in which light-induced changes in the level of active GAs have been detected in relatively large harvested portions, and even in whole seedlings. Our results do demonstrate that such changes do not occur in pea seedlings.

Additional evidence that light does not act through changes in GA_1 level in pea has come from the investigation of the light responses of the *sln* mutant, and comparison of *sln* (or GA_1-treated wild-type) plants with *lv*. The *sln* mutant retains a normal response to continuous W and R (Fig. 3) and to EOD–FR treatment (Fig. 4), both of which are lacking in *lv* (Nagatani et al. 1990). In addition, GA_1 application and the *lv* mutation have different effects on a range of important developmental traits, including rate of node development, the cellular basis for stem elongation, and leaf morphology (Fig. 5, Table 2). Furthermore, GA application has been shown to delay flowering in short-day conditions, thus increasing the size of the flowering response to photoperiod (Reid et al. 1977), while *lv* has the opposite effect (Weller and Reid 1993). These results show that the *lv* phenotype cannot be mimicked by application of GA to wild-type plants. Taken together with results from quantification of GAs in the *lv* mutant, they indicate that there is no causal relationship in either direction between increased GA_1 levels and reduced response to phyB, thus addressing the uncertainty raised by Chory (1993).

The reduction in GA_{20} levels consistently observed in dark-grown (Fig. 2; Ross and Reid 1989; Ross et al. 1992) and *lv* plants (Fig. 2) is too great to be attributed solely to differences in the proportion of leaf to stem tissue in the harvested apical portion and may indicate an effect of light on GA_{20} metabolism, also reported by Sponsel (1986) and Ross and Reid (1989). The significance of this change is not clear. However, preliminary investigation of $[^3H]GA_{20}$ metabolism in *lv* plants indicated that the rate of GA_1 production was not substantially altered (Reid and Ross, 1988). The reduced levels of GA_{20} thus do not reflect an increased rate of 3β-hydroxylation and are therefore unlikely to be directly related to the increased elongation. Furthermore, it has recently been shown that the response to exogenous GA_1 is increased when both the production (endogenous GA_{20} 3β-hydroxylation) and metabolism (2β-hydroxylation) of GA_1 are blocked by prohexadione (Kamiya et al. 1991; Nakayama et al. 1992), suggesting that the action of GA_1 is not related to the rate of its production or metabolism, nor, therefore, to its rate of turnover. This is in contrast to the suggestion that in *Brassica*, GA turnover as well as GA levels may mediate the photocontrol of elongation (Rood et al. 1990a, 1993).

Studies involving GA_1 application have previously indicated that darkness and *lv* confer increased responsiveness to exogenous GA_1 (Kende and Lang 1964; Reid 1988; Reid and Ross 1988), producing a shift in the dose-response curve for GA_1. In this study, we have further explored the relationship between light, gibberellins and elongation using the GA_1-deficient mutants *na*, *ls* and *le* and the double mutants *na lv*, *ls lv* and *le lv*. Our results suggest that etiolated plants are also more responsive to their *endogenous* GA_1 content, while use of the double mutants confirms the similarity of the effects of *lv* and etiolation in this respect (Figs. 6, 7).

These results can be extended with evidence accu-

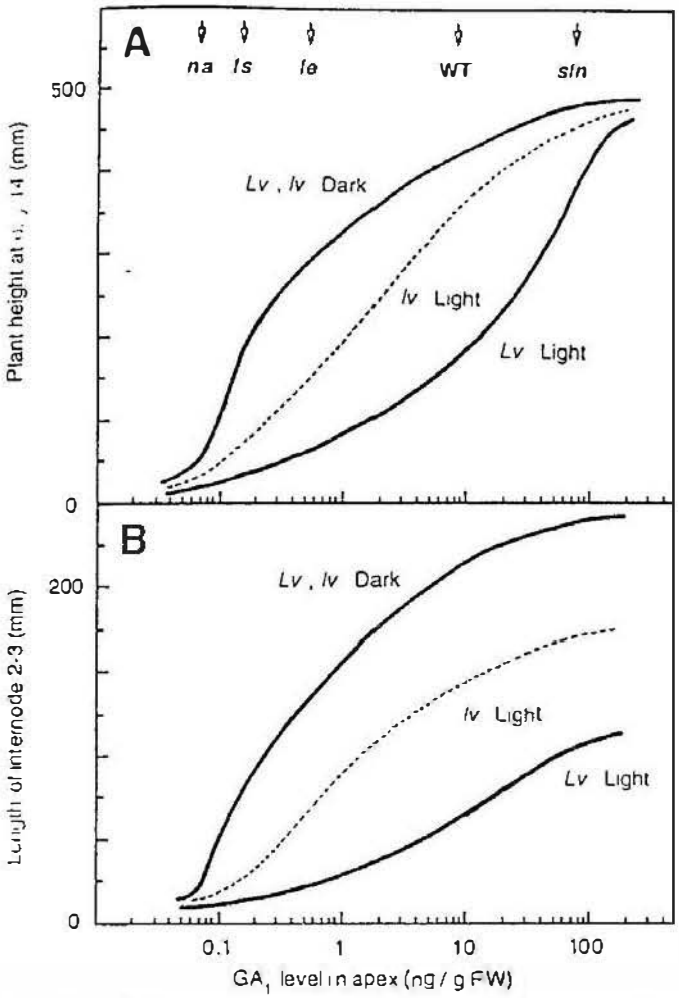

Fig. 8A, B. Suggested model for the relationship between apical GA_1 level and A plant height at 14 d and B length between nodes 2 and 3 in pea seedlings, showing the effects of darkness and the *lv* mutation on this relationship. These diagrams have been assembled from a number of experiments variously utilizing GA_1-deficient mutants (*na, ls, lh', le*), the doubly recessive *lh ls* mutant, the GA_1-overproducing *sln* mutant, double-mutant combinations of these mutations with *lv*, and application of GA_1 and the GA-biosynthesis inhibitor paclobutrazol. The *arrows* indicate the approximate level of GA_1 in the mutants and the wild type (*WT*)

mulated from GA_1-application studies, and studies of the *sln* mutant and extremely GA_1-deficient double-mutant combinations (such as *lh' ls*; Swain 1993) to provide a model for the interaction between light, *lv*, endogenous GA_1 content and stem elongation (Fig. 8A) or internode length (Fig. 8B) over a range in GA_1 content spanning more than three orders of magnitude. Etiolation and the *lv* mutation shift the dose-response curve to the left, as well as increasing the maximal internode length, describing a relationship between GA_1 and light that is largely additive. Thus, the level of GA_1 does appear to be important, in that GA_1 may play a permissive role in expression of the etiolated or the *lv* elongated phenotype. However, the retention of normal light responses at saturating levels of GA_1 show that changes in GA_1 level are not sufficient or indeed necessary to explain phyB elongation responses.

This is consistent with the suggestion that light may act by modifying or constraining some aspect of the GA-signal transduction pathway, in general agreement with the suggestion of Nick and Furuya (1993). However, the major differences in the action of GA_1 and *lv* (i.e. the fact that the *sln* and *lv* mutants are not phenocopies of each other) indicate that such an interaction is likely to occur well down the respective transduction pathways of light and GA_1. The fact that etiolation and active GAs have some similar effects on cell-wall biophysical parameters (Behringer et al. 1990, Kigel and Cosgrove 1991) and microtubule orientation (Nick and Furuya 1993) suggests that the two pathways may intersect prior to microtubule arrangement.

While a role for substances other than gibberellins (e.g. auxins, Jones et al. 1991; novel growth inhibitors, Noguchi and Hashimoto 1991) in the control of elongation by light has been suggested, definitive proof of such a role has yet to be demonstrated. Further examination of the actions of these substances and of their interactions with gibberellins may be useful in understanding the way in which the effects of light on elongation are mediated within the plant.

Conclusion. In this study, we have demonstrated that compared with light-grown wild-type plants, neither etiolation nor the phyB-response mutation *lv* substantially alters the levels of the major active endogenous gibberellin (GA_1) in expanding tissue of pea seedlings. It thus appears that phyB does not control elongation through alteration of GA_1 levels. This is consistent with the evidence that *lv* and the GA_1-overproducing mutant *sln* are not true phenocopies. Furthermore, there is no evidence that the rate of GA_1 production or GA_1 metabolism per se (and hence GA_1 turnover) are important determinants of stem elongation in pea. However, we have shown that etiolation and the *lv* mutation both increase the *responsiveness* of the wild-type plant to GA_1. Interactions between light treatment, the *lv* mutant, and various other mutants altering stem elongation are consistent with the hypothesis that light acts in part by constraining GA-signal transduction at a relatively late stage. We present a model, based on both the present study and our previous work, which integrates changes in GA_1 level and changes in the elongation response to light.

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New *lv* Mutants of Pea Are Deficient in Phytochrome B[1]

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The *lv-1* mutant of pea (*Pisum sativum* L.) is deficient in responses regulated by phytochrome B (phyB) in other species but has normal levels of spectrally active phyB. We have characterized three further *lv* mutants (*lv-2*, *lv-3*, and *lv-4*), which are all elongated under red (R) and white light but are indistinguishable from wild type under far-red light. The phyB apoprotein present in the *lv-1* mutant was undetectable in all three new *lv* mutants. The identification of allelic mutants with and without phyB apoprotein suggests that *Lv* may be a structural gene for a B-type phytochrome. Furthermore, it indicates that the *lv-1* mutation results specifically in the loss of normal biological activity of this phytochrome. Red-light-pulse and fluence-rate-response experiments suggest that *lv* plants are deficient in the low-fluence response (LFR) but retain a normal very-low-fluence-rate-dependent response for leaflet expansion and inhibition of stem elongation. Comparison of *lv* alleles of differing severity indicates that the LFR for stem elongation can be mediated by a lower level of phyB than the LFR for leaflet expansion. The retention of a strong response to continuous low-fluence-rate R in all four *lv* mutants suggests that there may be an additional phytochrome controlling responses to R in pea. The kinetics of phytochrome destruction and reaccumulation in the *lv* mutant indicate that phyB may be involved in the light regulation of phyA levels.

The importance of the regulatory photoreceptor phytochrome in the control of plant development is well known. Phytochrome is a chromoprotein consisting of a linear tetrapyrrole chromophore linked to an apoprotein of 114 to 130 kD (Quail, 1991; Furuya, 1993; Terry et al., 1993). Regulatory properties of the receptor derive from its property of reversible interconversion between two molecular forms, Pr and Pfr. It has recently been established that several distinct molecular species of the photoreceptor are present in higher plants. The apoprotein components of these forms differ in immunological reactivity (Abe et al., 1985; Somers et al., 1991; Wang et al., 1991) and primary structure (Abe et al., 1989) and have been shown to be the products of a small family of divergent genes, designated *PHYA-E* in Arabidopsis (Sharrock and Quail, 1989; Clack et al., 1994).

Functional differentiation within the phytochrome family has now been clearly demonstrated by the characterization of mutants specifically deficient in either phytochrome A (phyA) or phytochrome B (phyB). Study of phyA-deficient mutants in Arabidopsis and tomato has shown that phyA controls the response to high-irradiance FR in etiolated seedlings but appears to have little role in mediation of responses to continuous R or W (Nagatani et al., 1993; Parks and Quail, 1993; Whitelam et al., 1993; van Tuinen et al., 1995). Mutants deficient in phyB are known in Arabidopsis (*hy3* = *phyB*; Nagatani et al., 1991; Somers et al., 1991; Reed et al., 1993), cucumber (*lh*; López-Juez et al., 1992), and *Brassica* (*ein*; Devlin et al., 1992). The ma_3^R mutant of *Sorghum* is also deficient in a stable phytochrome, possibly phyB (Childs et al., 1992). These mutants show strongly reduced inhibition of elongation by W and R. However, etiolated seedlings of phyB-deficient Arabidopsis mutants respond normally to continuous FR, indicating that at this stage of Arabidopsis development phyA and phyB have discrete roles in the mediation of elongation responses to FR and R. PhyB-deficient mutants are also characterized by a reduced "shade avoidance" response to FR-enriched W, failure to elongate in response to treatment with EOD-FR, and reduced flowering response to photoperiod (López-Juez et al., 1990; Goto et al., 1991; Nagatani et al., 1991; Somers et al., 1991; Whitelam and Smith, 1991; Devlin et al., 1992). The clear effects of phyB deficiency in the mature plant suggest that this phytochrome, unlike phyA, has an important role throughout the life of the plant (Reed et al., 1993).

The pea *lv-1* mutant has many of the characteristics of a phyB-deficient mutant. Etiolated seedlings show reduced inhibition of elongation by R and W but retain a normal response to FR (Nagatani et al., 1990). Mutant plants lack a normal elongation response to EOD-FR and low R:FR ratio and are earlier flowering under short (noninductive) photoperiods (Weller and Reid, 1993). However, an initial investigation of phytochrome in *lv-1* indicated that the mutant possessed normal levels of both phyA and phyB and normal levels of spectrophotometrically detectable phyto-

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Abbreviations: EOD, end-of-day; FR, far-red light; LFR, low fluence response; phyA, phytochrome A; phyB, phytochrome B; R, red light; TBST, Tris-buffered saline-Tween; W, white light; WT, wild type.

chrome in extracts from both etiolated and light-grown plants, leading to the conclusion that the mutation might affect phytochrome signal transduction (Nagatani et al., 1990). Alternatively, it might specifically affect the biological function of the photoreceptor, as has been reported for mutations in the *PHYA* gene of Arabidopsis (Dehesh et al., 1993). In this case, it should be possible to identify other *lv* mutants that are deficient in the phyB apoprotein. We have recently examined three additional *lv* mutants, and we report here that all are severely deficient in phyB.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The pure lines of *Pisum sativum* L. used in this study are held in the collection at the University of Tasmania, Hobart, Australia. The origins of *lv* mutant lines NEU3 and R83 from cv Sparkle, lines 232^{+} and 232^{-} from the cross NEU3 × cv Torsdag, line Wt10895 from cv Paloma, and line 80m from Hobart line 80 have been described previously (Reid and Ross, 1988; Weller et al., 1992; Weller and Reid, 1993). The *lv* mutant alleles carried in these lines will subsequently be referred to as *lv-1* (NEU3, $L232^{-}$), *lv-2* (R83), *lv-3* (Wt10895), and *lv-4* (L80m). All four alleles are currently in the process of transfer to a uniform genetic background, but in the present study, the original mutants were used, and the parental lines were included as the corresponding WT line in each case. In the case of mutant *lv-3*, isogenic WT and mutant lines were not available, and because the original line showed signs of substantial background heterogeneity, we did not include *lv-3* in any of the physiological experiments reported here, except for the initial light quality screen.

Plants for experiments in Figures 1 and 2 were grown at 20°C in 140-mm pots or plastic boxes in a 1:1 (v/v) mixture of dolerite chips and vermiculite topped with potting soil. For all other experiments, plants were grown at 25°C in pure vermiculite in plastic trays.

Light Sources

The light sources for the light quality and dominance experiments (Figs. 1 and 2) were as follows: R, Tungsram (Budapest, Hungary) 40-W red fluorescent tubes unfiltered; W, Thorn (Sydney, Australia) 40-W cool-white fluorescent tubes; and FR, Thorn 100-W incandescent globes filtered through 10 cm of running water and one sheet of FR Perspex (FRF-700; Westlake Plastics, Lenni, PA). The R and FR sources used in pulse, fluence-rate-response, and phytochrome-reaccumulation experiments and the green light source used during manipulation of plants and extracts were identical with those used by Nagatani et al. (1993). Where necessary, the fluence rate of the R source was reduced by filtering through finely perforated metal plates. The W and supplementary FR sources used in the R:FR experiment were identical with those described by López-Juez et al. (1995).

Immunoblotting

Approximately 0.2 g of 1-cm apical segments from 5-d-old etiolated seedlings were harvested on ice, frozen in liquid nitrogen, and homogenized with approximately 0.02 g of insoluble PVP in 0.2 mL of phytochrome extraction buffer (50% [w/v] ethylene glycol, 20 mM $NaHSO_3$, 52 mM 2-mercaptoethanol, and 4 mM PMSF) containing additional protease inhibitors leupeptin (2 μg/mL), pepstatin (1 μg/mL), and aprotinin (2 μg/mL) and 4 mM iodoacetamide (López-Juez et al., 1992). The homogenate was centrifuged at 15,000*g* for 10 min. To this point, the extract was maintained at less than 4°C. The crude supernatant was collected, vortexed together with a 1:1 volume of 2× electrophoresis sample buffer, and allowed to stand for 2 min at 90°C. Extracts were subjected to SDS-PAGE in a 6.5% gel, and proteins were electroblotted onto a nylon filter (Fineblot; Atto, Tokyo, Japan) in 100 mM Tris, 192 mM Gly, and 25% (v/v) methanol. Membranes were blocked in a series of TBST solutions, essentially as described by López-Juez et al. (1992), with the exception that rabbit IgG was omitted from the second wash. Membranes were incubated with the primary antibody in TBST containing 1% (w/v) fat-free milk powder, for 1 h (phyA) or 2 h (phyB antibodies) at room temperature. After washing with TBST, membranes were incubated with a 1:5000 dilution of anti-mouse IgG-alkaline phosphatase conjugate (Protoblot, Promega) for 1 h at room temperature and stained according to the manufacturer's instructions.

Monoclonal Antibodies

The anti-pea phyA monoclonal antibody mAP5 has been described previously (Nagatani et al., 1984). Monoclonal mAP11 (Konomi et al., 1987) was raised against a pea type-2 phytochrome purified from green plants (Abe et al., 1985). The mAT5 and mAT2 antibodies were raised against a C-terminal fragment of tobacco phyB and shown to cross-react with the mAP11-purified type-2 pea phytochrome (phyB) but not with mAP5-purified type-1 phytochrome (phyA; López-Juez et al., 1992). We used all three of these phyB-specific antibodies in this study.

Photoreversible Phytochrome Measurement

Phytochrome was measured with a dual-wavelength recording spectrophotometer (model 557; Hitachi, Tokyo, Japan) using 730- and 800-nm measuring beams. For each measurement, eight apical segments (15 mm in length) from 5-d-old etiolated seedlings were harvested on ice, diced, and packed gently into a precooled stainless steel cuvette, presenting a light path of about 4 mm. Phytochrome was photoconverted using saturating irradiances of R (45 s) and FR (90 s).

RESULTS AND DISCUSSION

Photocontrol of Elongation

Like the originally isolated *lv-1* mutant, all three new *lv* mutants were substantially longer than their respective WT

lines in continuous R and W but were no longer under FR (Fig. 1). In general, there is considerable variation in the relative response of standard pea cultivars to monochromatic light, and variation in the response of the WT lines used in this study made it difficult to assess the relative strengths of the different mutant alleles. However, on the same genetic background (cv Sparkle) *lv*-2 was clearly less severe than *lv-1*, as previously reported (Reid and Ross, 1988). The *lv-1* and *lv-4* mutations appeared similar in relative severity under R. In addition, a difference in the response of *lv-1* to high-fluence W was apparent. The other three mutants were approximately the same length in R as in W and longer in FR, whereas *lv-1* was longer in W than in R or FR (Fig. 1; Nagatani et al., 1990). Two of the *lv* mutants, *lv-1* and *lv-4*, have recently been mapped to the same location in linkage group 6 (Weller and Murfet, 1994), thus making it unlikely that this difference in the *lv-1* phenotype might in part result from translocation or from an otherwise altered position of the gene. However, it cannot yet be ruled out that the difference may represent a background effect.

Dominance

The F_1 plants from crosses between mutants *lv-1*, *lv-2*, and *lv-4* and their respective WT lines were in each case significantly longer than the corresponding WT (Fig. 2). Although these differences were relatively small, they do indicate incomplete dominance of the WT allele over the mutant alleles. On the basis of the results in Figure 2, the degree of dominance of the *Lv* allele over the *lv-1*, *lv*-2, and *lv-4* alleles was 85, 67, and 74%, respectively. This is in keeping with previous reports that phytochrome-deficient mutants of Arabidopsis show a partially dominant phenotype (Koornneef et al., 1980; Whitelam et al., 1993). It has been reported that the elongation phenotype of phyB-overexpressing transgenic Arabidopsis varies in a linear manner with phyB copy number (Wester et al., 1994). However,

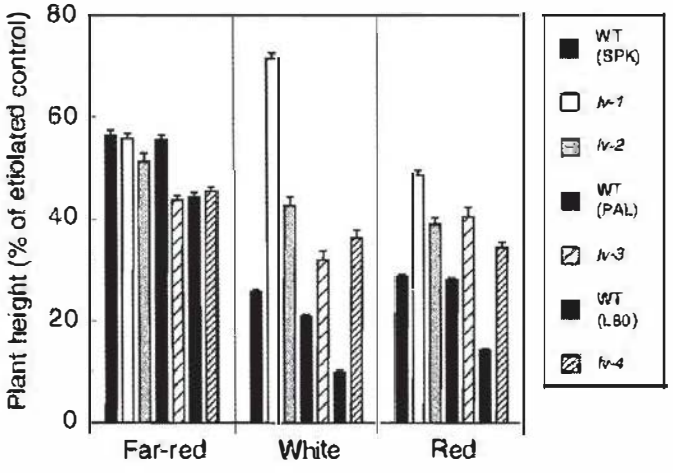

Figure 1. Stem elongation of *lv* mutants and corresponding WT lines under continuous FR (8 μmol m^{-2} s^{-1}), W (140 μmol m^{-2} s^{-1}), and R (20 μmol m^{-2} s^{-1}). Total plant height was measured on d 14 after sowing and expressed as a percentage of the height of etiolated plants. Bars indicate SE, n = 10 to 12.

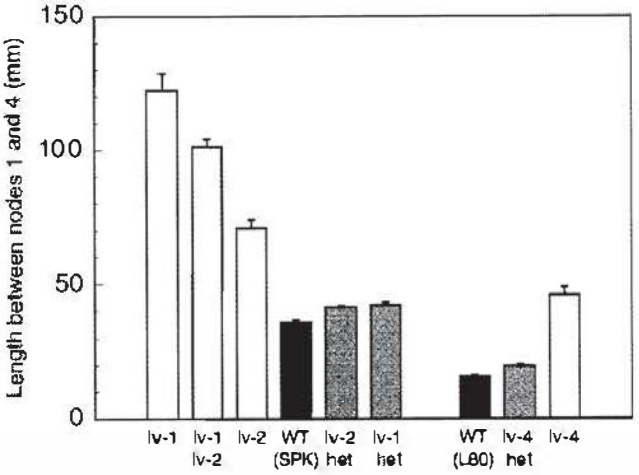

Figure 2. The stem length between nodes 1 and 4 of homozygous *lv-1*, *lv-2*, and *lv-4* mutant lines, corresponding WT lines, heterozygous *Lv lv-1*, *Lv lv-2*, and *Lv lv-4* plants (*lv-1* het, *lv-2* het, and *lv-4* het), and heterozygous *lv-1 lv-2* plants grown under continuous W (140 μmol m^{-2} s^{-1}). Bars indicate SE, n = 10 to 12.

heterozygous *Lv lv* plants were still much shorter than predicted on the basis of such a relationship, because in this case the WT gene would be expected to show no dominance. On the other hand, no dominance of either the *lv-1* or the *lv*-2 allele was seen in *lv-1 lv*-2 heterozygous plants (Fig. 2).

Phytochrome Levels

The anti-pea phyA antibody mAP5 detected a band of approximately 121 kD at uniform intensity in extracts from WT and all *lv* mutants (Fig. 3a). Although mAP11-immunopurified pea phyB was previously reported to migrate as a single band of 115 kD (Abe et al., 1989), the antibody detected two bands of about 116 kD in crude and partially enriched extracts from pea embryonic axes (Konomi et al., 1987). Figure 3b shows that in crude extracts from etiolated WT and *lv-1* seedlings, mAP11 also recognized two bands, at 117 and 115 kD, consistent with these previous reports. The same two bands were recognized by another anti-phyB monoclonal antibody, mAT5 (Fig. 3b). However, corresponding bands were not detected in extracts from *lv*-2, *lv-3*, or *lv-4* plants by mAP11 or mAT5 (Fig. 3b) despite uniform nonspecific staining. An identical result was also obtained with the weakly staining mAT2 antibody (data not shown). The absence of both the 115- and 117-kD band in the mutants also provides evidence that the two bands derive from a single phytochrome and not from cross-reactivity to more than one phytochrome species.

These results confirm the normal presence of phyB-like apoprotein in *lv-1* reported by Nagatani et al. (1990) and clearly show this phytochrome to be absent in the *lv*-2, *lv-3*, and *lv-4* mutants or present below the detection limit of about 3% established by dilution of WT extracts (data not shown). The limited amount of amino acid sequence data available for this phytochrome (69 residues; Abe et al., 1989) indicates a greater similarity to B-type phytochromes (86% to phyB, 81% to phyD) than to phyA (65%), C (61%),

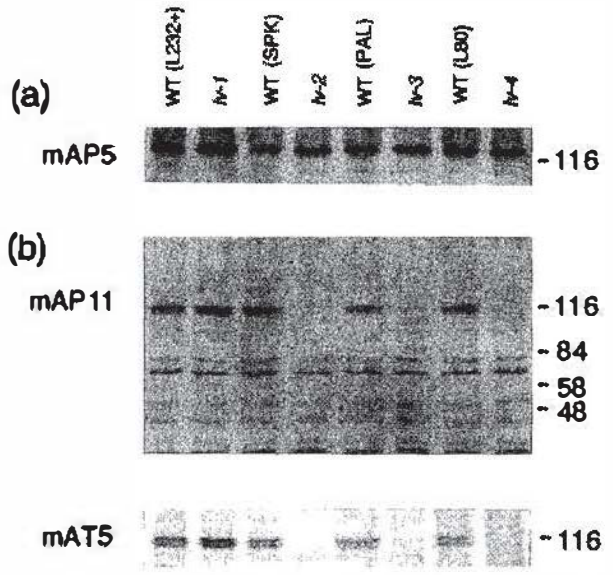

Figure 3. Immunoblot detection of phyA and phyB apoprotein in crude extracts of etiolated WT and *lv* mutant plants. Each lane contained extract equivalent to 2.5 mg fresh weight. The positions and molecular masses (kD) of prestained markers (Sigma) are indicated at the right of the diagram. a, Detection of phyA by monoclonal antibody mAP5. b, Detection of phyB by monoclonal antibodies mAP11 and mAT5.

or E (70%) of Arabidopsis (Sharrock and Quail, 1989; Clack et al., 1994). Because the existence of more than one B-type phytochrome in pea has yet to be demonstrated, we will refer operationally to the missing phytochrome as phyB, although the exact molecular identity of this phytochrome is not yet known. Nevertheless, the identification of phytochrome-deficient *lv* mutants now makes it clear that the original *lv-1* mutation must specifically alter the function of the photoreceptor without detectable alteration to its size or spectral activity and suggests that *Lv* may be a phytochrome structural gene. However, whether the *lv-1* mutation imparts additional, abnormal activity to the phyB molecule is not clear. In any case, this result suggests that molecular analysis of the *lv-1* mutation may be useful in identifying a residue(s) critical for phyB function. The *lv-1* mutant is therefore similar to the *phyA-103* mutant of Arabidopsis. This mutant possesses normal levels of spectrally active phyA that lacks biological activity because of a point mutation in the *PHYA* gene causing a substitution at a highly conserved residue ($Gly^{727} \rightarrow Glu$) in the encoded protein (Dehesh et al., 1993; Parks and Quail, 1993). A point mutation in the Arabidopsis *PHYB* gene causing the substitution $His^{283} \rightarrow Tyr$ (Reed et al., 1993; mutant *phyB-4*) also appears to eliminate normal phyB function without effect on levels of the apoprotein, although it is not known whether this molecule retains spectral activity.

Responses to R

The previously reported *lv* mutant phenotype (Nagatani et al., 1990; Weller and Reid, 1993) is consistent with the clear role for phyB in the mediation of elongation responses to continuous R, R:FR ratio, and EOD-FR treatment established by studies of the phyB-deficient *phyB* mutant of Arabidopsis (Koornneef et al., 1980; Nagatani et al., 1991; Whitelam and Smith, 1991; McCormac et al., 1993; Reed et al., 1994). This phenotypic syndrome is also shared by mutants of cucumber and *Brassica*, each deficient in a phyB-like phytochrome (Devlin et al., 1992; López-Juez et al., 1992). It appears that the primary function of phyB is the sensing of R, with EOD-FR and R:FR effects deriving from a reduction in the proportion of phyB as Pfr (Smith, 1994). It is well known that different components of the seedling response to R can be distinguished on the basis of FR reversibility or dependence on fluence rate (Mancinelli, 1994). To identify those components that are altered in the *lv* mutants, we examined in greater detail the responses of *lv* mutants to R. The data presented in Figure 4 are those for *lv-1* and *lv-2* in the same genetic background. The *lv-1* mutant is not, strictly speaking, deficient in phyB. However, under the R conditions used, it behaves similarly to the *lv-4* mutant (results not shown), which is severely deficient in the phyB apoprotein (Fig. 3).

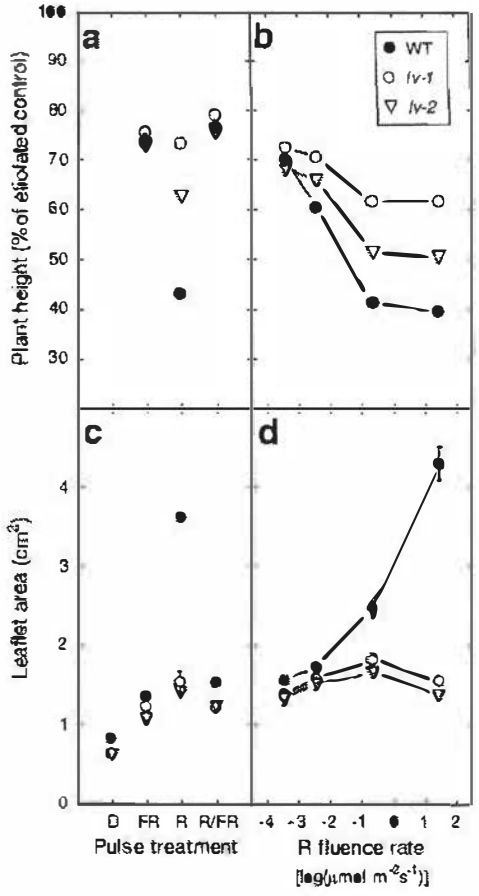

Figure 4. Responses to R pulses (a and c) and to fluence rate of continuous R (b and d) in the *lv-1* and *lv-2* mutants. In the R pulse experiments, plants were given saturating pulses of R (17 μmol m^{-2} s^{-1}, 10 min), FR (12 μmol m^{-2} s^{-1}, 15 min), or R followed by FR (R/FR) at 4-h intervals for 10 d after sowing or maintained in complete darkness (D). Total plant height (a and b) was measured and expressed as a percentage of the height of etiolated plants. Leaflet area (c and d) was estimated as the product of the length and width of a single leaflet from the second true foliage leaf (node 4). Bars indicate SE, $n = 12$ to 15.

Saturating R pulses given every 4 h to WT plants resulted in strong inhibition of elongation relative to dark-grown plants. This inhibition was partially reversible by FR, to the level of plants given FR pulses alone (Fig. 4a). The *lv-1* mutant appeared virtually null for this FR-reversible component, whereas the the *lv-2* mutant was clearly leaky. The residual, nonreversible component was retained in both mutants (Fig. 4a). In a directly comparable experiment, we examined the R-fluence-rate response in *lv-1* and *lv-2* mutants (Fig. 4b). Elongation of WT plants was inhibited by continuous R in a fluence-rate-dependent manner over a range in fluence rate of approximately 10^{-3} to 10^{+1} μmol m^{-2} s^{-1}. Mutants *lv-1* and *lv-2* both showed a reduction in this response. Although we were not able to obtain fluence rates lower than about 4×10^{-4} μmol m^{-2} s^{-1}, the convergence of response curves for WT and *lv* mutant plants suggests that the mutants respond normally to R at lower fluence rates. Furthermore, this response to continuous very-low-fluence R is similar in extent to the non-FR-reversible component seen in the pulse experiment (Fig. 4a).

These results are for the most part consistent with those obtained in similar experiments with the Arabidopsis *phyB* mutant (McCormac et al., 1993; Reed et al., 1994). They indicate that in pea phyB mediates the classic LFR for stem elongation, induced by R pulses or low-fluence-rate continuous R. Furthermore, the threshold and saturation fluence rates (approximately 4×10^{-4} and 4×10^{-1} μmol m^{-2} s^{-1}) for the fluence-rate-dependent component of the response to continuous R appear approximately similar between the two species (Fig. 4b; Reed et al., 1994). However, our results also highlight possible differences between the pea and Arabidopsis systems, in the form of responses that are clearly apparent in pea but that are absent or undetectable in Arabidopsis. First, a very-low-fluence-rate-dependent response is clearly evident in pea, in which seedling stem elongation may be inhibited 20 to 30% by brief FR or R/FR pulses or by very-low-fluence-rate continuous R. However, in Arabidopsis, these same treatments appear to have little or no effect (McCormac et al., 1993, figs. 1b and 2; Reed et al., 1994, figs. 1 and 2). Further experiments will be necessary to determine whether this response corresponds to the previously described very-low-fluence response (Mancinelli, 1994) or is simply a more sensitive LFR. Second, inhibition of hypocotyl elongation by continuous R at a high-fluence rate is completely lacking in the Arabidopsis *phyB* mutant (McCormac et al., 1993; Parks and Quail, 1993; Reed et al., 1994, table IV), whereas *lv* plants, although longer than the WT under R, still show a strong inhibition of elongation relative to plants grown in darkness (Figs. 1 and 4b), particularly during the first 2 to 3 d after emergence.

Although none of the *lv* mutants has conclusively been shown to be null, plants carrying the most severe allele, *lv-1*, appear completely lacking in the FR-reversible component of the response to R pulses and yet retain a response to continuous high-fluence R (cf. Fig. 4, a and b). This is also true for the *lv-4* allele (results not shown), which has no detectable phyB apoprotein (Fig. 3). The failure to find clear residual effects of R on hypocotyl elongation in phyB null mutants of Arabidopsis has led to the conclusion that phyB may be the only phytochrome mediating elongation responses to R (McCormac et al., 1993; Parks and Quail, 1993). Our results indicate that this is not the case in pea and raise the question of which phytochrome(s) mediate the responses to R retained in the *lv* mutant. It is becoming increasingly clear from recent work with Arabidopsis that the non-FR-reversible very-low-fluence-rate components of the R-induced inhibition of hypocotyl elongation, seed germination, and *CAB* induction are controlled by phyA (Reed et al., 1994), and it seems highly probable that this is also the case in pea. On the other hand, the existence of a response to low-fluence-rate R additional to that controlled by phyB has little precedent in other species, although some evidence has recently been provided by studies of the *tri* mutant of tomato (Koornneef and Kendrick, 1994). This mutant lacks a phyB-like protein and is insensitive to R, but in contrast to both Arabidopsis *phyB* and pea *lv* mutants, the R insensitivity of *tri* plants is restricted to the first 2 d of exposure to light; mature light-grown plants retain normal responses to EOD-FR treatment and have an essentially WT appearance (Koornneef and Kendrick, 1994). In addition, it has recently been reported that *tri* mutants are deficient in one of the two B-type phytochromes present in tomato (Kendrick et al., 1994). These results suggest that the inhibition of elongation by continuous R may be controlled by at least two phytochromes mediating two overlapping but temporally distinct responses: one early and temporary and another later and persistent. Given the strong response to R retained in all *lv* mutants (Fig. 1), pea may possibly be a useful species for the identification of mutants lacking this response.

The results for leaflet expansion (Fig. 4, c and d) are essentially similar to those for stem elongation. The *lv* mutants lack the FR-reversible component of the response to R pulses (Fig. 4c) and the fluence-rate-dependent component of the response to continuous R (Fig. 4d). The WT plants show a small very-low-fluence-rate-dependent response for leaflet expansion, and this is retained in both mutants. Whereas *lv-1* is clearly less responsive than *lv-2* with respect to stem elongation (Fig. 4, a and b), leaf expansion responses in *lv-1* and *lv-2* plants are very similar, indicating that the level of phyB present in *lv-2*, although sufficient to inhibit elongation, is not sufficient to promote leaflet expansion. More generally, this suggests that there may be differences in the threshold level of phyB required for induction of the various developmental processes controlled by this phytochrome.

Response to R:FR Ratio

The addition of supplementary FR to background W causes an increase in stem elongation and a decrease in leaflet/cotyledon area (the so-called "shade-avoidance" response) in many plants, including pea. We reported previously that the *lv-1* mutant shows a negative response to FR supplementation (i.e. a decrease in elongation and an increase in leaflet area; Weller and Reid, 1993). However, this earlier study involved the use of unfiltered incandescent

globes as the FR source, introducing additional spectral differences between the low and high R:FR conditions used. We therefore re-examined the R:FR ratio response of the *lv-1*, *lv-2*, and *lv-4* mutants using two identical W sources and supplementation with monochromatic FR. Under these conditions, *lv-1* plants again showed inhibition of elongation and increased leaflet area under low R:FR, as did *lv-4* plants (Fig. 5). Interestingly, mutant plants carrying the weaker *lv-2* allele were shorter than *lv-1* plants under both low and high R:FR (Fig. 5) but showed similar leaf expansion under both conditions, in keeping with the comparison from the pulse and fluence-rate experiments (Fig. 4).

Phytochrome Reaccumulation

High levels of *PHYA* mRNA and phyA apoprotein are present in dark-grown pea seedlings and are rapidly depleted on exposure to R. Subsequent return to darkness results in an eventual reaccumulation of both mRNA and apoprotein (Clarkson and Hillman, 1967; Otto et al., 1984). The reaccumulation of *PHYA* mRNA is hastened by FR treatment, whether given directly after the R treatment or after an intervening dark period of up to 16 h (Furuya et al., 1991). These results indicate that the regulation of phyA abundance involves a rapid initial destruction on conversion to Pfr and a subsequent persistent repression of further phyA synthesis that may be alleviated by FR treatment, in a manner consistent with control by a stable phytochrome. This pattern of control has also been reflected in studies of *PHYA* expression in oat (Colbert et al., 1985; Lissemore and Quail, 1988) and regulation of phyA levels under a variety of conditions in several other species (Schäfer, 1978; Carr-Smith et al., 1994; Quail, 1994).

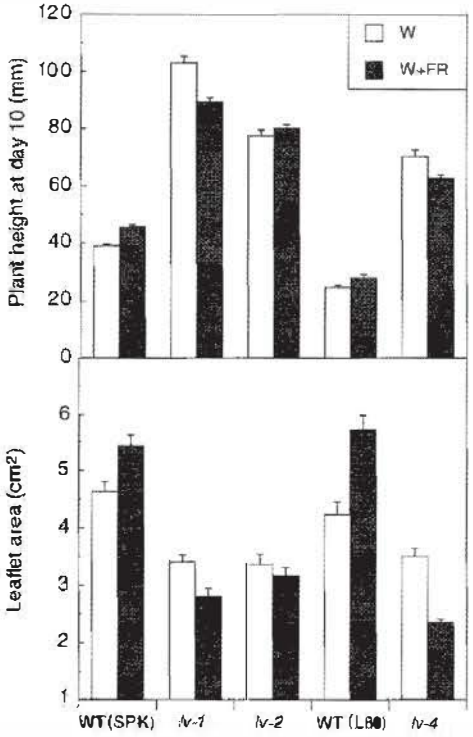

Figure 5. Response of *lv-1*, *lv-2*, and *lv-4* plants to R:FR ratio. Plants were grown for 10 d under continuous cool-white fluorescent light (40 μmol m^{-2} s^{-1}), either with (R:FR = 0.66) or without (R:FR = 5.44) supplementary FR. Leaflet area was estimated as the product of the length and width of a single leaflet from the second true foliage leaf. R:FR was calculated as the ratio of photon fluence rates in the 655- to 665- and 725- to 735-nm wavebands. Bars indicate SE, n = 12 to 15.

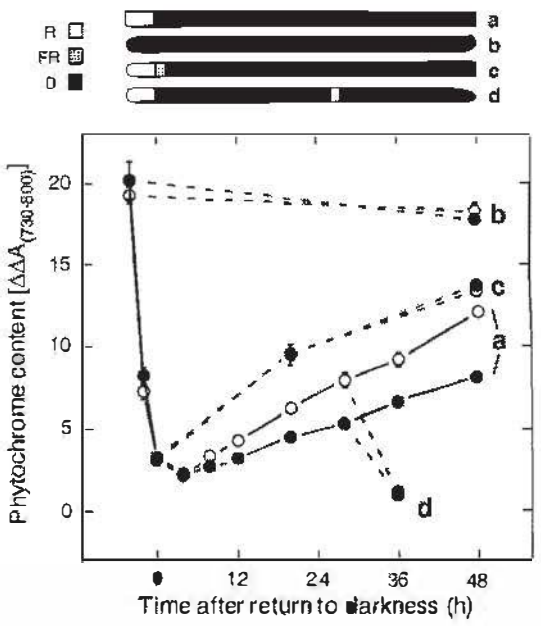

Figure 6. Destruction and reaccumulation kinetics of spectrophotometrically detectable phytochrome in WT (●) and *lv-4* mutant plants (○). Plants were grown for 5 d in complete darkness and then subjected to light treatments. a, Four hours of continuous R (17 μmol m^{-2} s^{-1}) followed by return to darkness; b, maintained in continuous dark; c, 4 h of R, 15 min of FR (12 μmol m^{-2} s^{-1}), and then returned to darkness; d, 4 h of R, 23 h of dark, 1 h of R, and 7 h of dark. These light treatments are indicated diagrammatically at the top of the figure. Each point for the basic reaccumulation time course (a) is the mean of at least four replicates (from separate plantings) with three determinations per replicate, whereas all other points are the means of two such replicates. Bars indicate SE; where not visible, bars are smaller than the plot symbols.

To examine whether this stable phytochrome might be phyB, we investigated the effects of the *lv-4* mutation on the destruction and subsequent reaccumulation of spectrophotometrically detectable phytochrome (Fig. 6). The *lv-4* mutation was chosen because it was the most severe of the three *lv* mutations causing deficiency in the phyB apoprotein. Phytochrome levels in etiolated WT and *lv-4* plants were similar and showed similar rates of depletion in R to about 15% of the level in etiolated plants after 4 h of R. WT plants returned to darkness after 4 h of R showed a gradual reaccumulation of phytochrome to about 45% after 48 h. However, *lv-4* plants showed more rapid reaccumulation, to about 66% after 48 h (Fig. 6a). A pulse of FR given immediately following R treatment also hastened reaccumulation in both WT and *lv-4* plants, to about 75% after 48 h in each case (Fig. 6c). However, clear differences in morphology became apparent between WT and *lv* mutant plants grown under this regime, and because in vivo spectrophotometric measurements are sensitive to changes in

the optical properties of the sample, we tested the effect of an additional 1 h of R treatment given 24 h after the initial transfer to darkness (Fig. 6d). This treatment re-depleted phytochrome in both lines to the same level but had no detectable effect on apical morphology in either genotype, indicating that the apparent difference in phytochrome content after 24 h of darkness was not due to a morphological difference.

The *lv* mutant thus shows a clear reduction in the R/FR-reversible control of phytochrome reaccumulation. This is in agreement with the previously proposed action of a stable phytochrome in this response (Furuya et al., 1991) and in the control of phyA accumulation generally (Hilton and Thomas, 1987). In addition, this result demonstrates clearly the involvement of phyB in the regulation of phyA levels. Further experiments and use of a proven phyB-null mutant will be necessary to determine whether phyB acts alone in mediating this response.

CONCLUSION

Three new *lv* mutants show reduced response to R and W and are deficient in immunochemically detectable phyB. Physiological experiments with the mutants show that this phytochrome controls responses in the classic LFR mode of phytochrome action, such as the R/FR-reversible inhibition of elongation by R pulses in etiolated seedlings and the fluence-rate-dependent response to continuous low-fluence R. These results indicate that the function of phyB in pea and in Arabidopsis is very similar. The *lv* mutants retain a normal very-low-fluence-rate-dependent response, consistent with control of this response by phyA. The *lv* mutants also retain a partial response to high-fluence R, suggesting the possible action of another R-sensing phytochrome. A reduction in FR-reversible control of phytochrome reaccumulation in etiolated *lv* seedlings suggests that phyB is at least a component of the stable phytochrome pool previously reported to be involved in this response.

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The Phytochrome-Deficient *pcd1* Mutant of Pea Is Unable to Convert Heme to Biliverdin IXα

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We isolated a new pea mutant that was selected on the basis of pale color and elongated internodes in a screen under white light. The mutant was designated *pcd1* for phytochrome chromophore deficient. Light-grown *pcd1* plants have yellow-green foliage with a reduced chlorophyll (Chi) content and an abnormally high Chi *a*/Chi *b* ratio. Etiolated *pcd1* seedlings are developmentally insensitive to far-red light, show a reduced response to red light, and have no spectrophotometrically detectable phytochrome. The phytochrome A apoprotein is present at the wild-type level in etiolated *pcd1* seedlings but is not depleted by red light treatment. Crude phytochrome preparations from etiolated *pcd1* tissue also lack spectral activity but can be assembled with phycocyanobilin, an analog of the endogenous phytochrome chromophore phytochromobilin, to yield a difference spectrum characteristic of an apophytochrome–phycocyanobilin adduct. These results indicate that the *pcd1*-conferred phenotype results from a deficiency in phytochrome chromophore synthesis. Furthermore, etioplast preparations from *pcd1* seedlings can metabolize biliverdin (BV) IXα but not heme to phytochromobilin, indicating that *pcd1* plants are severely impaired in their ability to convert heme to BV IXα. This provides clear evidence that the conversion of heme to BV IXα is an enzymatic process in higher plants and that it is required for synthesis of the phytochrome chromophore and hence for normal photomorphogenesis.

INTRODUCTION

The phytochromes are a family of chromoprotein photoreceptors with a well-established and important role in the mediation of plant developmental responses to light (Kendrick and Kronenberg, 1994). The spectral characteristics of phytochrome derive from an interaction between an apoprotein and a covalently bound linear tetrapyrrole chromophore (Lagarias and Rapoport, 1980). Holophytochrome biosynthesis therefore requires the convergence of two separate pathways, one for synthesis of the apoprotein and another for synthesis of the chromophore precursor, phytochromobilin (PΦB). Several distinct forms of the phytochrome apoprotein exist, and these are products of a small gene family designated *PHYA* to *PHYE* in Arabidopsis (Sharrock and Quail, 1989; Clack et al., 1994). Assembly of apophytochrome with PΦB is an autocatalytic process that is thought to occur in the cytoplasm (Lagarias and Lagarias, 1989; Terry and Lagarias, 1991). There is no evidence for the involvement of any other protein in phytochrome assembly. Because assembly to phycocyanobilin (PCB), a structural analog of PΦB, proceeds at the same rate for both PHYA (Li and Lagarias, 1992) and PHYB (Kunkel et al., 1993) apoproteins, it is likely that at least these phytochromes (and possibly PHYC as well; Quail et al., 1995) use the same chromophore.

Figure 1 shows the proposed pathway for PΦB synthesis in higher plants, which has been developed on the basis of metabolism studies (Elich and Lagarias, 1987; Elich et al., 1989; Terry et al., 1995) and by analogy with the pathway for the synthesis of related bilins in algae (Beale, 1993; Terry et al., 1993b). Synthesis of PΦB appears to occur entirely in the plastid (Terry and Lagarias, 1991; Terry et al., 1993b), where it is derived from 5-aminolevulinic acid and is synthesized via a pathway that branches from the synthetic pathway for chlorophyll (Chl). The first committed step for PΦB synthesis is thought to be the oxygenation of heme to biliverdin (BV) IXα, which is subsequently reduced to 3(*Z*)–PΦB. 3(*Z*)–PΦB is further isomerized to 3(*E*)–PΦB, which is considered to be the natural chromophore precursor (Cornejo et al., 1992; Terry et al., 1995). Although much progress has been made recently, understanding of this pathway in higher plants is still far from complete. For example, although ferrochelatase has been cloned (Smith et al., 1994) and PΦB synthase activity has been characterized (Terry and Lagarias, 1991; Terry et al., 1995), direct evidence for heme oxygenase and PΦB isomerase activities has yet to be demonstrated (Terry et al., 1993b, 1995).

Since the advent of a mutant-based approach to plant photomorphogenesis, many mutants that alter phytochrome function have been identified and characterized (Koornneef and Kendrick, 1994). These include mutants deficient in specific

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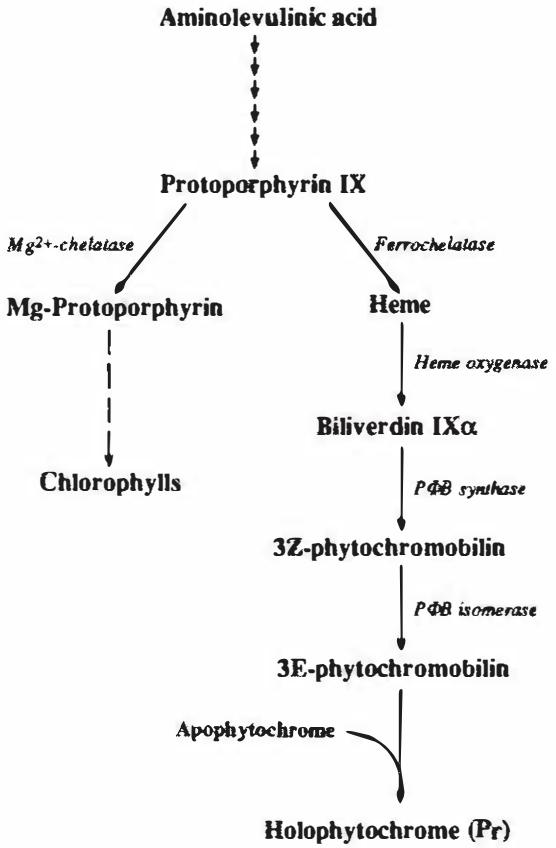

Figure 1. Proposed Pathway for Phytochrome Chromophore Biosynthesis in Higher Plants.

The phytochrome chromophore is synthesized from aminolevulinic acid via a pathway that branches from the pathway for Chl synthesis by the chelation of Fe^{2+} rather than Mg^{2+} to protoporphyrin IX.

phytochromes. Study of these mutants has established that phyA, the light-labile phytochrome abundant in etiolated seedlings, is involved specifically in the mediation of seedling responses to far-red light (FR) (Nagatani et al., 1993; Parks and Quail, 1993; Whitelam et al., 1993; van Tuinen et al., 1995a), whereas the light-stable B-type phytochromes appear to control responses to red light (R) (Somers et al., 1991; Devlin et al., 1992; López-Juez et al., 1992; van Tuinen et al., 1995b; Weller et al., 1995).

A third class of mutant, deficient in response to both R and FR, is represented by the long hypocotyl *hy1*, *hy2*, and *hy6* mutants of Arabidopsis (Koornneef et al., 1980; Chory et al., 1989). In view of what is known from phyA- and phyB-specific mutants, the coincidence of R and FR insensitivity in *hy1*, *hy2*, and *hy6* suggests that the lesions in these mutants affect the activity of both phyA and phyB. Etiolated seedlings of *hy1*, *hy2*, and *hy6* have strongly reduced levels of spectrally active phytochrome but wild-type or nearly wild-type levels of PHYA apoprotein (Koornneef et al., 1980; Chory et al., 1989; Parks et al., 1989), suggesting that phytochrome apoprotein is synthesized normally in these mutants but for some reason is not converted into a spectrally active holoprotein. The most plausible explanation, that the mutants are deficient in the phytochrome chromophore, is further supported by the demonstration that exogenously supplied BV can fully or partially restore a wild-type phenotype in the *hy1*, *hy2*, and *hy6* mutants (Parks and Quail, 1991; Nagatani et al., 1993).

Similar mutants are also known in tomato. Like *hy1*, the *aurea* (*au*) and *yellow-green-2* (*yg-2*) mutants are insensitive to both R and FR and have strongly reduced levels of spectrally active phytochrome (Koornneef et al., 1985; Parks et al., 1987; Kendrick et al., 1994). Attempts to rescue the *au* mutant by feeding chromophore precursors or structural analogs have proven unsuccessful so far (Kendrick et al., 1994). However, the phenotypic similarity of *au* to the chromophore-deficient Arabidopsis mutants and epistasis of *au* and *yg-2* over a constitutively expressed *PHYA* transgene are consistent with the hypothesis that both *au* and *yg-2* are blocked in phytochrome chromophore biosynthesis (Kendrick et al., 1994).

Thus, among the several candidates for mutations that block phytochrome chromophore synthesis, only the *hy1*, *hy2*, and *hy6* mutants clearly have been shown to be chromophore deficient (Parks and Quail, 1991), and their sites of action remain to be determined. Identification of the precise steps blocked by these mutants will contribute greatly to further elucidation of the details of the pathway for PΦB synthesis and may provide confirmation of the enzymatic nature of certain poorly characterized steps. In addition, *hy1*, *hy2*, *hy6*, and *au* are all widely used as phytochrome-deficient controls in genetic, physiological, and biochemical studies (e.g., Neuhaus et al., 1993; Millar et al., 1995), and further knowledge about the sites of action and potential secondary effects of such mutations therefore will assist in interpretation of these data.

We are using pea as an additional model species for the investigation of the genetic basis of phytochrome action, including phytochrome chromophore biosynthesis. This species has a number of advantages over Arabidopsis and tomato for biochemical studies, including the ease of obtaining large amounts of dark-grown tissue. We have recently isolated a number of pea mutations that appear to affect the pathway for chromophore biosynthesis, and in this study we characterize one such mutant, *pcd1* (for *p*hytochrome chromophore *d*eficient). This mutant is very similar in phenotype to the *au* mutant of tomato and the *hy1* mutant of Arabidopsis. Here, we report that the *pcd1* mutant is deficient in phytochrome chromophore synthesis and that this deficiency results from an inability of mutant plants to convert heme to BV IXα.

RESULTS

Isolation of the *pcd1* Mutant

Pea mutant line S2-14 was selected for its yellow-green foliage and elongated internodes relative to parental cultivar Solara plants in an M_2 generation grown in the glasshouse under a

natural photoperiod. In addition to pale foliage, mutants have slightly elongated internodes, show reduced branching, and flower slightly later than wild-type plants grown under the same conditions. The phenotypes of wild-type and S2-14 plants grown in continuous white light (W) are shown in Figure 2. A cross of S2-14 to cultivar Solara yielded an entirely wild-type F_1 and an F_2 segregation of 49 wild-type plants to 15 pale mutants ($X^2_{(3:1)} = 0.083$, $P > 0.7$). A cross of S2-14 to a standard wild-type line, cultivar Torsdag, returned an F_2 segregation of 82 wild-type plants to 28 pale mutants ($X^2_{(3:1)} = 0.012$, $P > 0.9$). Perfect cosegregation of all characteristic aspects of the S2-14 mutant phenotype in close accord with a 3:1 ratio indicated that this pleiotropic phenotype most probably results from a recessive mutation at a single locus, which we have designated *pcd1*.

pcd1 Mutant Shows Altered Photomorphogenesis

Figure 3 shows the responses of *pcd1* to broad-band monochromatic light. The *pcd1* mutant was dramatically elongated under FR, appearing completely insensitive to FR in terms of both elongation and rate of development (Figures 3A and 3B). Because phyA is the predominant phytochrome mediating responses to continuous FR (Reed et al., 1994; van Tuinen et al., 1995a), this result indicates that *pcd1* is strongly deficient in phyA activity. Dark-grown *pcd1* and wild-type plants were the same height (Figure 3B) and showed no other visible difference, indicating that expression of the *pcd1*-conferred phenotype is dependent on light and therefore that the mutation is truly photomorphogenic in nature. A marked reduction in the response of *pcd1* to R was also apparent, although the mutant appeared to retain some sensitivity to R (Figure 3B). This reduction in sensitivity to R is more severe in *pcd1* than in phyB-deficient *lv* mutants (Weller et al., 1995), suggesting that *pcd1* shows strongly reduced activity of phyB and at least one other R-sensing phytochrome. In contrast, the sensitivity of *pcd1* to W and to blue light was only slightly reduced compared with the wild type (Figures 2 and 3).

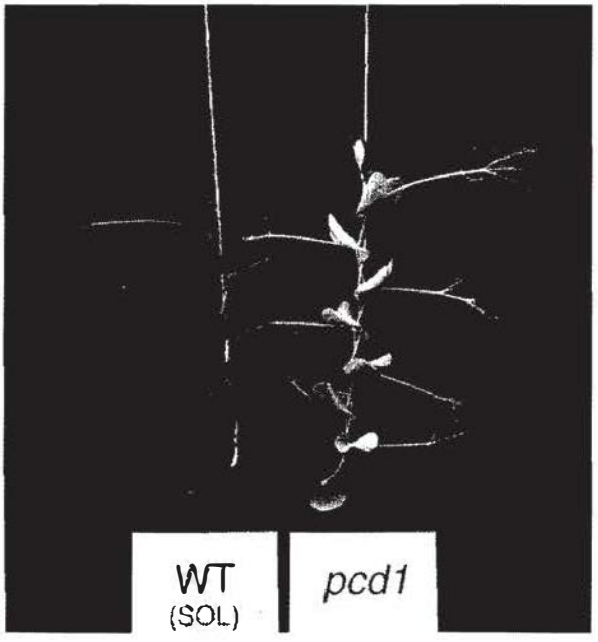

Figure 2. Phenotype of the *pcd1* Mutant.

Plants were grown at 20°C in continuous fluorescent W (150 μmol m^{-2} sec^{-1}) for 30 days. The cultivar Solara (WT [SOL]) background carries Mendel's dwarfing *le* mutation and the *afila* mutation (homeotic conversion of leaflets to tendrils).

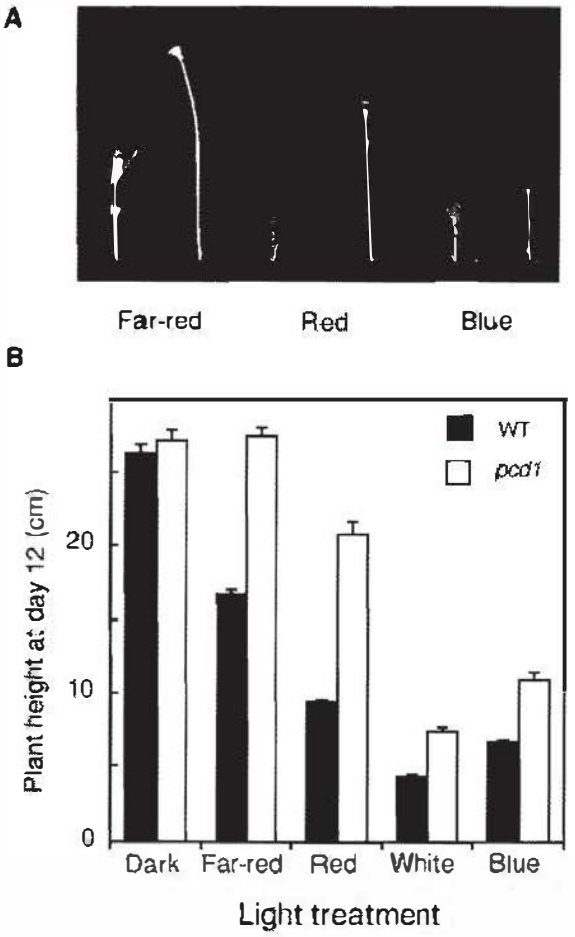

Figure 3. Phenotype of the *pcd1* Mutant in Monochromatic Light.

Seedlings were grown in the dark or under continuous FR (8 μmol m^{-2} sec^{-1}), R (20 μmol m^{-2} sec^{-1}), blue light (10 μmol m^{-2} sec^{-1}), or fluorescent W (150 μmol m^{-2} sec^{-1}) for 12 days after sowing.
(A) Representative wild-type (left of each pair) and *pcd1* seedlings.
(B) Total plant height (n = 12 to 15). Error bars represent SE. WT, wild type.

We also tested the response of *pcd1* to end-of-day (EOD)–FR treatment. Figure 4 shows that wild-type plants responded strongly to EOD-FR treatment, which leads to increased internode elongation throughout the growth of the plant. In contrast, over the very early internodes, *pcd1* seedlings showed a greatly reduced reponse to EOD-FR. However, with increasing age,

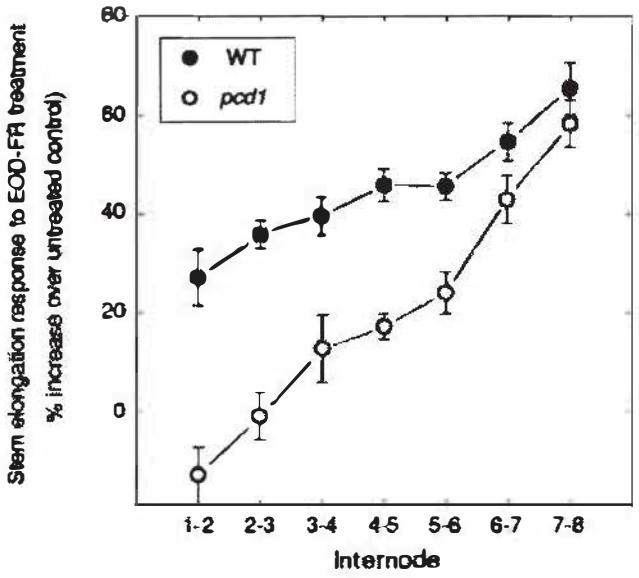

Figure 4. Elongation Response of *pcd1* to EOD-FR Treatment.

Plants were grown for 3 weeks at 20°C in a 12-hr light/dark cycle with or without 20 min of EOD-FR treatment (8 μmol m^{-2} sec^{-1}). The absolute increase in length in response to EOD-FR was 70 ± 4 cm for the wild type (WT) and 48 ± 5 cm for *pcd1* over internodes 1 to 8 (n = 12 to 15). Error bars represent SE.

mutant seedlings gradually recovered the ability to respond, to the extent that newly produced internodes in 3-week-old *pcd1* plants showed the same relative response as those of wild-type plants. It has been shown that phyB is the principal phytochrome controlling the elongation response to EOD-FR, because phyB-deficient mutants of Arabidopsis (Nagatani et al., 1991), Brassica (Devlin et al., 1992), cucumber (López-Juez et al., 1992), and pea (Nagatani et al., 1990) all lack this response. The response of the *pcd1* mutant is different from that of phyB-deficient *lv* mutants, which show no such recovery of EOD-FR response with age (Nagatani et al., 1990). This result shows that *pcd1* is strongly deficient in phyB activity at the seedling stage but recovers phyB activity as the plant matures.

Mature W-grown *pcd1* plants have a yellow-green phenotype (Figure 2), and we noticed that this varied considerably, depending on the conditions under which the plants were grown. We grew *pcd1* plants under a number of different W regimens and found that under all conditions, *pcd1* caused a reduction in Chl content and a substantial increase in the Chl *a*/Chl *b* ratio. This was most pronounced in short photoperiods and least pronounced in plants grown in continuous light. Representative effects of the *pcd1* mutation on foliar Chl levels are shown in Table 1.

These aspects of the *pcd1*-conferred phenotype are also common to photomorphogenic mutants of Arabidopsis (*hy1*, *hy2*, and *hy6*) and tomato (*au* and *yg-2*). All of these mutants are severely deficient in spectrophotometrically detectable phytochrome (Koornneef et al., 1980, 1985; Chory et al., 1989). We therefore examined the phytochrome content of *pcd1* in more detail.

Spectrophotometric and Immunochemical Analysis of Phytochrome in the *pcd1* Mutant

Figure 5A shows representative difference spectra for in vivo phytochrome phototransformation in standard samples of etiolated wild-type and *pcd1* tissue. Although samples from wild-type plants contained phytochrome routinely giving a signal of 40 to 50 units (one unit is 10^{-3} $\Delta\Delta A_{660-730\ nm}$), the signal in *pcd1* samples was below the detection limit of the spectrophotometer, which was ~0.3 units. Etiolated *pcd1* plants therefore contain <1% of the spectrophotometrically detectable phytochrome present in the wild type.

We also examined the phytochrome apoprotein content of the *pcd1* mutant. Immunoblot analysis of crude protein extracts showed that etiolated *pcd1* plants have normal levels of the PHYA apoprotein (Figure 5B). In addition, the depletion of PHYA apoprotein seen in response to irradiation of wild-type seedlings with 4 hr of R was not observed for the *pcd1* mutant (Figure 5B). As the depletion of PHYA after R is dependent on conversion to Pfr, this suggests that PHYA in the *pcd1* mutant does not undergo photoconversion. Both of these results are consistent with the suggestion that phytochrome in *pcd1* plants lacks a chromophore.

Table 1. Chl Content and Chl *a*/Chl *b* Ratio of the *pcd1* Mutant

Photoperiod (hr)	Genotype	Chl Content (mg g fresh weight^{-1})[a] Chl *a*	Chl *b*	Total Chl	Chl *a*/*b*
24	WT[b]	1.67 ± 0.12	0.52 ± 0.04	2.19 ± 0.16	3.2
	pcd1	1.41 ± 0.16	0.17 ± 0.25	1.58 ± 0.18	8.4
8	WT	1.50 ± 0.12	0.38 ± 0.03	1.88 ± 0.14	3.9
	pcd1	0.25 ± 0.01	0.03 ± 0.01	0.28 ± 0.01	12.0

[a] Values are expressed as the mean ± SE of six samples. Tissue samples were taken from stipules because the wild type has no true leaflets.
[b] Wild type.

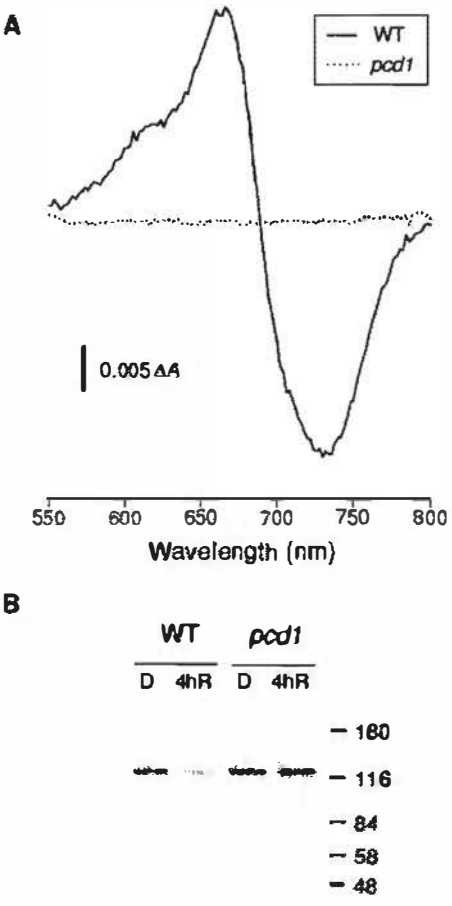

Figure 5. Phytochrome Content of *pcd1* Seedlings.

(A) Differencespectrumfor in vivo phytochrome phototransformation (Pfr-Pr) in etiolated wild-type (WT) and *pcd1* seedlings.

(B) PHYA apoprotein content in crude protein extracts from wild-type and *pcd1* seedlings grown in complete darkness (D) or given a 4-hr R treatment (4hR; 17 μmol m^{-2} sec^{-1}) before harvest. The positions and molecular masses (kilodaltons) of prestained markers (Sigma) are indicated. Lanes were loaded on an equivalent fresh weight basis. The PHYA apoprotein was detected using an anti-pea phyA monoclonal antibody mAP5 (Nagatani et al., 1984).

pcd1 Mutant Is Deficient in the Phytochrome Chromophore

One possible explanation for the deficiency in phytochrome spectral activity in *pcd1* is that the apophytochrome has undergone a modification that prevents assembly in vivo. To address this, we attempted to assemble apophytochrome extracted from etiolated *pcd1* seedlings. We partially purified the phytochrome apoprotein from *pcd1* plants in the presence of PCB. PCB is a structural analog of the native phytochrome chromophore PΦB and derives from the light-harvesting chromoprotein C-phycocyanin. Previous reports have demonstrated that PCB can substitute for PΦB in the assembly of a spectrally and biologically active phytochrome holoprotein (Elich and Lagarias, 1989; Parks and Quail, 1991) and that this assembly is an autocatalytic process, requiring only apophytochrome and PCB (Lagarias and Lagarias, 1989).

Figure 6 shows that the apophytochrome in *pcd1* extracts can assemble with PCB to give a spectrally active phytochrome, when PCB is included in the extraction buffer. The difference spectrum for this holophytochrome has absorption peaks in the R (652 nm) and FR (716 nm) that are blue shifted by 16 and 14 nm, respectively, relative to the corresponding peaks for phytochrome in wild-type samples. These are very close to the peak values previously reported for a pea apophytochrome A-PCB adduct (Deforce et al., 1993). We typically recovered 65 to 80% of wild-type spectral activity in *pcd1* extracts incubated with PCB, based on equivalent tissue fresh weight. Apophytochrome that was extracted from *pcd1* tissue in the absence of PCB, but subsequently assembled with PCB added to the partially purified extract, resulted in a holoprotein with spectral characteristics identical to those shown in Figure 6 (data not shown). However, yields were considerably lower (~25%), indicating that the apoprotein was not stable during the extraction procedure. These results clearly show that the loss of spectral activity of the phytochrome in *pcd1* is due to a deficiency of the endogenous chromophore rather than incompetence of the apoprotein to bind chromophore.

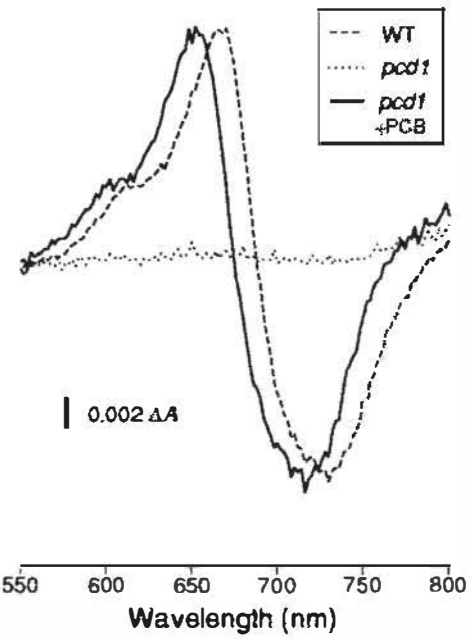

Figure 6. In Vitro Assembly of the *pcd1* Phytochrome Apoprotein with PCB.

Difference spectra for phytochrome (Pfr-Pr) in extracts from wild-type (WT), *pcd1*, and *pcd1* tissue extracted in the presence of 3 μM PCB (*pcd1*+PCB) are shown. The wild-type spectrum has been scaled down for ease of comparison, and the scale bar applies to *pcd1* and *pcd1*+PCB spectra only.

BV Restores Phytochrome Spectral Activity to *pcd1* in Vivo

We then examined the possibility that the *pcd1* mutation might lead in some other way to the failure or prevention of assembly in vivo. To test this hypothesis, we attempted to recover holophytochrome in vivo by feeding chromophore precursors to intact seedlings. It has been shown previously that both exogenously supplied PCB and BV can restore a wild-type level of spectrally active phytochrome and a wild-type light-grown phenotype in the chromophore-deficient mutant *hy1* of Arabidopsis (Parks and Quail, 1991). We tested whether the *pcd1* mutant might be rescued in a similar manner by supplying these chromophore precursors to germinating *pcd1* seed. Although we tried several methods of supplying PCB and BV to germinating seedlings, no restoration of light-induced inhibition of stem elongation or phytochrome spectral activity was seen (data not shown). However, because the ineffectiveness of these compounds could have resulted from problems with uptake from the growing medium, we attempted an alternative method of floating excised tissue segments in a solution of BV (Elich and Lagarias, 1987; Elich et al., 1989). Because pea shoot tissue treated in this way retains substantial amounts of BV that cannot be washed out, it was impossible to obtain valid in vivo measurements of phytochrome. We therefore partially purified phytochrome from BV-treated explants and measured spectral activity in vitro. PCB was not used because the possibility of assembly during the extraction procedure could not be excluded.

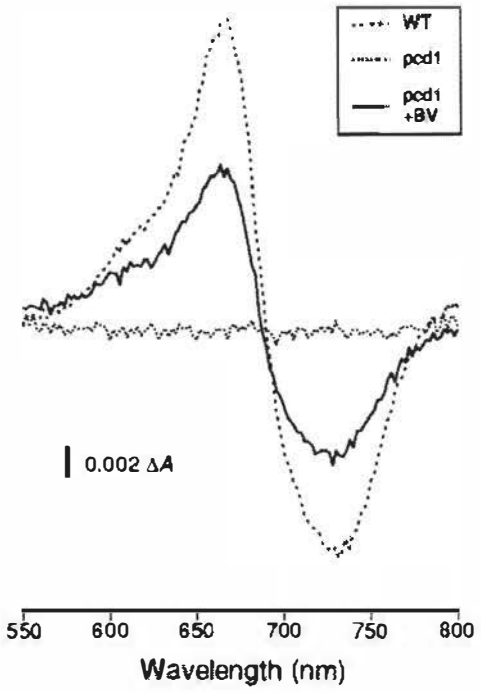

Figure 7. Recovery of Phytochrome Spectral Activity in *pcd1* Extracts Incubated with BV.

Difference spectra (Pfr-Pr) for phytochrome in extracts from wild-type (WT), *pcd1*, and BV-incubated *pcd1* tissue (*pcd1* + BV) are shown. Apical explant sections were floated in 300 μM BV or buffer alone for 6 hr at room temperature. The wild-type spectrum has been scaled down for ease of comparison. The scale bar applies to *pcd1* and *pcd1* + BV spectra only.

The difference spectrum of the newly synthesized holophytochrome is shown in Figure 7. This spectrum shows no substantial deviation from the spectrum of phytochrome extracted from wild-type tissue either in shape or in wavelength of the ΔA peaks (666 and 732 nm for both samples). Feeding of the natural BV isomer, BV IXα, gave an identical result (data not shown). The amount of holoprotein in *pcd1* after BV incubation was ~27% of that found in wild-type explants (based on equivalent tissue fresh weight); this recovery compares favorably with the value reported for gabaculine-treated oat coleoptiles (34%), using a higher concentration of BV (500 μM; Elich and Lagarias, 1987).

Because BV itself does not assemble in vitro with apophytochrome to give a spectrally active holoprotein (Li and Lagarias, 1992), the recovery of spectral activity in *pcd1* indicates that BV has been converted to PΦB and that assembly of holophytochrome has occurred in vivo. This result therefore shows that apophytochrome synthesis and assembly are normal in *pcd1* and that the mutant is deficient in phytochrome chromophore synthesis. In addition, the recovery of signal obtained with BV suggests that the block in chromophore synthesis imposed by *pcd1* occurs most probably before BV.

pcd1 Mutant Is Not Deficient in Heme

As shown in Figure 1, the immediate precursor of BV IXα in PΦB synthesis is thought to be heme. Because the *pcd1* mutation is sufficiently severe to prevent detectable PΦB synthesis, a block in the pathway before heme also would be expected to result in reduced heme levels. Previous attempts to recover phytochrome spectral activity from chromophore-depleted oat explants incubated with heme were reported to be unsuccessful (Elich et al., 1989); therefore, we did not attempt a similar experiment. Instead, to address whether the *pcd1* mutation might have its effect before heme formation, we quantitated total noncovalently bound heme (as an estimate of total cellular heme) in wild-type and *pcd1* seedlings. As shown in Table 2, we found no significant difference in the level of noncovalently bound heme in wild-type and *pcd1* seedlings. Hemes are also a component of all cytochromes and therefore are essential to the basic cellular functions of photosynthesis and respiration. Heme deficiency therefore might be expected to seriously compromise the general vigor of the plant. However, consistent with the heme quantitation data, *pcd1* plants are generally healthy plants and are not noticeably less vigorous than wild-type plants (see also Figure 2). These results, when taken together with the fact that *pcd1* plants are able to utilize BV (Figure 7), provide indirect evidence that the *pcd1* mutation may prevent the conversion of heme to BV IXα.

Table 2. Quantitation of Noncovalently Bound Heme in *pcd1* Seedlings

Genotype	Heme[a] nmol (g fresh weight)$^{-1}$	pmol (seedling)$^{-1}$
WT[b]	0.56 ± 0.15	67.2 ± 16.0
pcd1	0.55 ± 0.12	70.8 ± 14.3

[a] Values are expressed as mean ± SE of four replicate measurements.
[b] Wild type.

pcd1 Mutant Is Unable to Convert Heme to BV IXα

To address specifically the question of whether the *pcd1* mutant is unable to convert heme to BV IXα and PΦB, we used a recently developed assay system for the detection of PΦB (Terry et al., 1995). Isolated etioplasts were incubated with heme in the presence of an NADPH regenerating system, and the products were analyzed by reverse phase HPLC. Figure 8 shows that wild-type plastids converted heme to a major product with a retention time of ~11 min (trace C). The retention time of this peak is consistent with its identification as either BV IXα or 3(*Z*)-PΦB, which coelute in this solvent system (Terry et al., 1995).

To identify this peak unequivocally, we reexamined the products using a modified HPLC solvent system that can resolve BV IXα from 3(*Z*)-PΦB (Terry et al., 1995). This HPLC trace is shown in Figure 9A. Under these conditions, the original single peak resolved into two (Figure 9A, labeled *a* and *b*). Coinjection studies with authentic BV IXα and 3(*Z*)-PΦB samples provisionally identified peaks *a* and *b* (Figure 9A) as 3(*Z*)-PΦB and BV IXα, respectively (data not shown). The low yield of peak *b* precluded more rigorous identification, but peak *a* was purified and analyzed further.

Figure 9B shows an absorption spectrum of peak *a*, with BV IXα also shown for comparison. The spectrum of peak *a* is red shifted in comparison with BV IXα, consistent with its identification as 3(*Z*)-PΦB (Terry et al., 1995). For final verification of the identity of peak *a*, we attempted an assembly reaction with partially purified apophytochrome derived from the *pcd1* mutant. It has been demonstrated previously that 3(*Z*)-PΦB will assemble with apophytochrome, although it is not clear whether isomerization to the 3(*E*)-isomer is required for the assembly to proceed (Terry et al., 1995). As shown in Figure 9C, assembly of peak *a* with pea apophytochrome resulted in a holophytochrome with a difference spectrum that is almost identical to the wild-type pea phytochrome under these conditions (cf. Figures 6 and 7). This result confirms that the major product from the incubation of wild-type plastids with heme is 3(*Z*)-PΦB.

Incubation of wild-type plastids with heme also resulted in the synthesis of a product with a retention time of ~19 min (Figure 8, trace C). This was identified as 3(*E*)-PΦB by coinjection with the authentic standard (data not shown). Because the major product was 3(*Z*)-PΦB, the additional synthesis of 3(*E*)-PΦB would be expected (Terry et al., 1995). Incubation of wild-type plastids in the absence of heme also resulted in the synthesis of 3(*Z*)-PΦB, though the yield was greatly reduced (Figure 8, trace A). This may reflect synthesis from endogenous chromophore precursors. Incubations of heme without plastids resulted in some coupled oxidation of the heme, leading to the appearance of a number of small peaks, one of which was identified as BV IXα (data not shown). When plastids isolated from *pcd1* seedlings were incubated with heme, the 3(*Z*)-PΦB peak was almost completely absent, and no 3(*E*)-PΦB was detected (Figure 8, trace D). No peaks were discernible from incubations of *pcd1* plastids in the absence of heme (trace B). These results clearly demonstrate that isolated *pcd1* plastids are unable to convert heme to 3(*Z*)-PΦB.

To confirm that *pcd1* can synthesize PΦB from BV IXα and that isolated plastids from *pcd1* are functionally active, we also examined these steps in the same plastid preparations. The metabolism of BV IXα to PΦB has previously been well characterized using oat etioplasts (Terry et al., 1995). As shown in

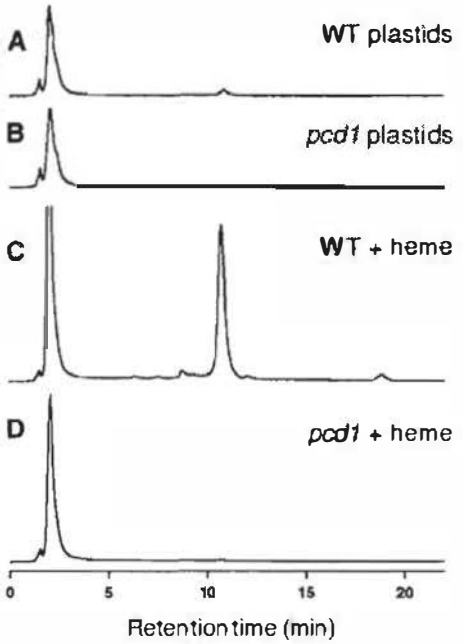

Figure 8. HPLC Analysis of Heme Metabolism in Wild-Type and *pcd1* Plastids.

(A) to **(D)** Plastids from etiolated seedlings of wild type (WT; traces **[A]** and **[C]**) and *pcd1* (**[B]** and **[D]**) were incubated in the presence (**[C]** and **[D]**) or absence (**[A]** and **[B]**) of 10 μM heme. Incubations were for 3 hr at 28°C and included an NADPH regenerating system. The final protein concentrations were 1.17 mg/mL for the wild type and 1.16 mg/mL for *pcd1*. The products were analyzed by reverse phase HPLC using a solvent system of ethanol–acetone–water–acetic acid (48:34:17:1 [v/v]), and absorbance was monitored at 380 nm. The absorbance scale for the samples containing plastids only (**[A]** and **[B]**) is half that of the samples from incubations with heme (**[C]** and **[D]**).

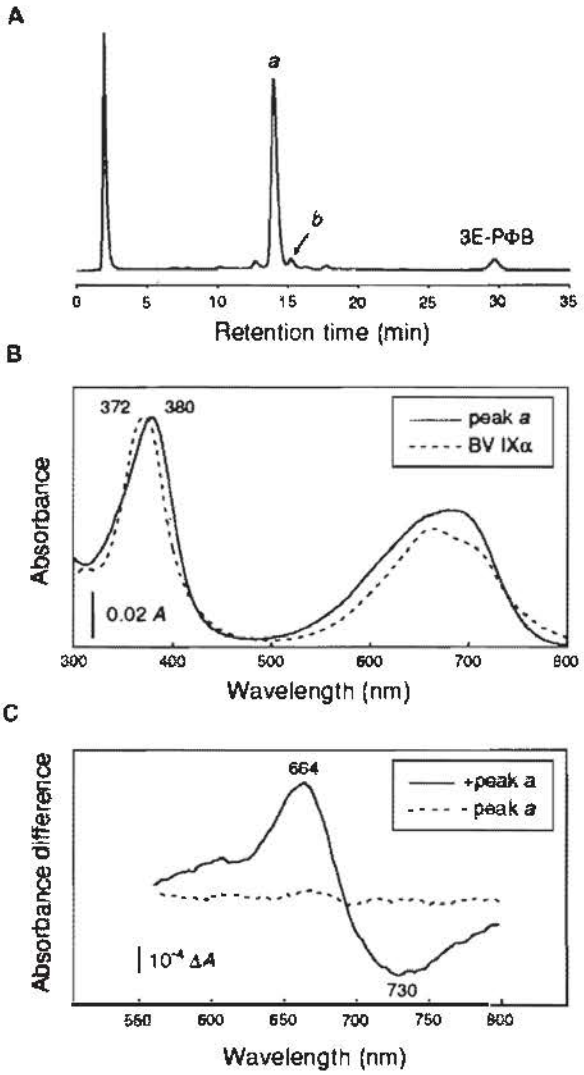

Figure 9. Analysis of the Major Product from the Incubation of Heme with Wild-Type Plastids.

(A) Reverse phase HPLC analysis of the products of incubating 10 μM heme with wild-type etioplasts. Incubations were for 3 hr at 28°C and also included an NADPH regenerating system. The final protein concentration was 0.84 mg/mL. The solvent system used was ethanol–acetone–100 mM formic acid (25:65:10 [v/v]), and absorbance was monitored at 380 nm. The identity of 3(*E*)-PΦB was confirmed by coinjection studies. Peak a was collected from two identical injections, one of which is shown.

(B) Absorbance spectra of peak a and BV IXα in acetonitrile–0.1% TFA (60:40 [v/v]).

(C) Phytochrome difference spectrum (Pfr-Pr) for apophytochrome assembled with peak a (+peak a). The phytochrome apoprotein was purified partially from *pcd1* tissue (10 g fresh weight), and the contents of peak a were added to the clarified extract to give a final bilin concentration of 0.4 μM (calculated using the absorption coefficient of 3(*Z*)-PΦB at 382 nm). Spectra were recorded after incubation of extracts on ice for 30 min and have been smoothed mathematically. The control extract (–peak a) was obtained from 2.5 g fresh weight.

Figure 10, both wild-type and *pcd1* plastids converted BV IXα to 3(*Z*)- and 3(*E*)-PΦB, the products confirmed by coinjection studies and absorption spectroscopy (data not shown). This result thus confirms that the conversion of BV IXα to 3(*Z*)- and 3(*E*)-PΦB is normal in *pcd1* and that this mutant is deficient specifically in the ability to synthesize BV IXα from heme.

DISCUSSION

pcd1 Is Unable to Convert Heme to BV IXα

Heme has been proposed to be the precursor of BV IXα in the biosynthesis of the phytochrome chromophore. The proposal is based on analogy with the pathway for phycobilin biosynthesis in the red alga *Cyanidium caldarium* (Beale, 1993; Terry et al., 1993b) and is supported by two pieces of experimental evidence. Isolated cucumber etioplasts can use heme, but not Mg–protoporphyrin, to synthesize PΦB detected by assembly to apophytochrome (Terry et al., 1993b). Second, application of an inhibitor of ferrochelatase, which is required for heme synthesis (see Figure 1), results in a reduction in spectrophotometrically detectable phytochrome in embryonic axes of pea (Konomi et al., 1993). In our assays, the major product after the incubation of heme with wild-type plastids was 3(*Z*)-PΦB, whereas BV IXα was present only in trace amounts (Figures 8 and 9). Plastids from *pcd1* seedlings were unable to synthesize 3(*Z*)-PΦB from heme (Figure 8), although the same plastid preparation could convert BV IXα to 3(*Z*)-PΦB

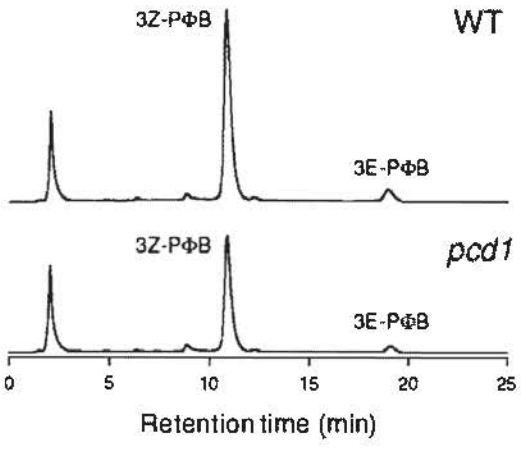

Figure 10. HPLC Analysis of BV IXα Metabolism by Wild-Type and *pcd1* Plastids.

Plastids from etiolated seedlings of the wild type (WT) and *pcd1* were incubated with 8 μM BV IXα. Incubations were for 3 hr at 28°C and included an NADPH regenerating system. The final protein concentrations were 1.17 mg/mL for the wild type and 1.16 mg/mL for *pcd1*. The products were analyzed by reverse phase HPLC, using a solvent system of ethanol–acetone–water–acetic acid (48:34:17:1 [v/v]), and absorbance was monitored at 380 nm. The identities of 3(*Z*)- and 3(*E*)-PΦB were confirmed by coinjection studies.

(Figure 10). Together with the demonstrations that *pcd1* seedlings are not heme deficient (Table 2) and that BV can restore holophytochrome to *pcd1* in vivo (Figure 7), these results clearly demonstrate that *pcd1* is unable to convert heme to BV IXα. These experiments therefore confirm the proposal that heme is an intermediate in the synthesis of the phytochrome chromophore.

We consider it probable that the *pcd1* mutation lies in the catalytic subunit of the enzyme accomplishing the conversion of heme to BV IXα. Unfortunately, there are no molecular probes available for this enzyme, and it was not possible to test this hypothesis directly. The enzymatic conversion of heme to BV IXα in higher plants is thought to be accomplished by the enzyme heme oxygenase, as it is in mammals and red algae (Cornejo and Beale, 1988; Maines, 1988; Beale, 1993). This activity has not been measured previously in higher plants, in part due to the problem of distinguishing facile, nonenzymatic-coupled oxidation of heme from the enzyme-catalyzed reaction (Terry et al., 1993b). The absence of this reaction in *pcd1* provides direct evidence for this step being enzyme catalyzed in higher plants. However, because the reaction proceeds to 3(*Z*)-PΦB, no further characterization has been performed, and the nature of the reaction from heme to BV IXα remains unknown. The reason that 3(*Z*)-PΦB, and not BV IXα, is the major product after the incubation of heme with isolated plastids is also unknown. However, the formation of BV IXα does not require PΦB synthase activity because, in mutants unable to synthesize 3(*Z*)-PΦB from BV IXα, incubation of heme results in BV IXα accumulation (M.J. Terry, J.L. Weller, J.B. Reid, and R.E. Kendrick, unpublished data).

Phenotype of the *pcd1* Mutant

Most of the observed aspects of the *pcd1*-conferred phenotype may be explained readily in terms of the deficiency in active phytochrome that results from the demonstrated block in PΦB synthesis. The mutant is clearly lacking in both phyA and phyB responses at the seedling stage and is at least partially deficient in a response to R, which is retained in phyB-deficient *lv* mutants (Weller et al., 1995). This implies that the PΦB deficiency results in a reduction in the level of activity of at least two and possibly all phytochromes in pea. One notable aspect of the *pcd1*-conferred phenotype is the gradual recovery of the phyB-mediated EOD-FR response. This suggests that more active phyB is present in the mature tissues of *pcd1* plants and thus implies that more PΦB has been synthesized. This might result from slight leakiness of *pcd1* or might reflect the presence of a *PCD1* homolog, which is expressed at higher levels in mature tissue. The location of the *pcd1* lesion after the branch point for Chl and PΦB synthesis shows that the pale phenotype of the mutant does not result from a direct impairment in Chl synthesis. However, phytochrome has well-documented effects on plastid development and on the synthesis of Chl (Kasemir, 1983) and Chl *a*/*b* binding proteins (Batschauer et al., 1994), and it is possible that one or more of these effects account for the pale phenotype. In addition, negative feedback effects of free heme on aminolevulinic acid synthesis have been proposed (Chereskin and Castelfranco, 1982). Although there was no difference in noncovalently bound heme (Table 2), it is possible that an accumulation of free heme in the *pcd1* mutant might occur, and this could also contribute to the chlorotic phenotype.

Relationship of *pcd1* to Other Mutants

Mutants similar in phenotype to *pcd1* have been identified in several other species. Mutants *au* and *yg-2* of tomato, *hy1*, *hy2*, and *hy6* of Arabidopsis, and *pew1* and *pew2* of *Nicotiana plumbaginifolia* are all pale, are elongated in FR and R, and have reduced levels of spectrally active phytochrome (Koornneef et al., 1980, 1985; Chory et al., 1989; Kraepiel et al., 1994). These mutants are all considered to be chromophore-deficient mutants, although this has been conclusively proven only for the *hy* mutants (Parks and Quail, 1991; Nagatani et al., 1993).

Although pale, the *au* and *yg-2* mutants are relatively healthy and vigorous, suggesting that these plants, like *pcd1*, are not deficient in heme (Kendrick et al., 1994). The lesions in *au* and *yg-2* are therefore also likely to affect steps after the formation of heme. Again, although the *hy1*, *hy2*, and *hy6* mutants have reduced Chl levels (Chory et al., 1989) and would be expected to have impaired photosynthetic ability, all three mutants are generally healthy. These mutants are therefore also unlikely to be deficient in heme, suggesting that they may be blocked after heme formation as well. Recent preliminary evidence from heme and BV IXα metabolism studies in *au* and *yg-2* has confirmed that both mutants are in fact blocked in PΦB synthesis after this point (M.J. Terry and R.E. Kendrick, unpublished data). The *hy1* mutant is clearly rescued by BV (Parks and Quail, 1991); therefore, it seems probable that this mutation may affect the same step as that altered by *pcd1*—preventing the conversion of heme to BV IXα. By the same reasoning, *hy2* has also been suggested to lie before BV (Parks and Quail, 1991). However, the incomplete rescue of *hy2* on BV (Parks and Quail, 1991) may indicate that the mutation is in fact after BV IXα. Such a mutation, if leaky, might still allow synthesis of significant amounts of PΦB in the presence of BV at a sufficiently high concentration. However, further investigation of the pathway for PΦB synthesis in *hy1* and *hy2* clearly is necessary to address these questions.

The results presented in this study identify the biochemical basis of the *pcd1*-conferred phenotype. They demonstrate conclusively that the phenotype results from impairment of a specific, committed step in phytochrome chromophore biosynthesis. Furthermore, they establish that heme is an intermediate in the synthesis of the phytochrome chromophore. The feasibility of addressing this kind of biochemical problem in pea highlights an advantage of this species for a mutant-based approach to understanding the function of phytochrome in higher plants. The present study should assist greatly in the investigation of similar mutants in other species less amenable to

biochemical investigation and should lead ultimately to a better understanding of phytochrome chromophore synthesis and its role in plant photomorphogenesis.

METHODS

Plant Material and Growth Conditions

The original *pcd1* (for *p*hytochrome *c*hromophore *d*eficient) mutant line S2-14 was derived from the pea (*Pisum sativum*) cultivar Solara at INRA Station de Genetique et d'Amelioration des Plantes (Versailles, France). Imbibed seeds of cultivar Solara were mutagenized by soaking in 4 mM ethyl methanesulfonate (EMS) for 4 hr at room temperature. The S2-14 mutant was identified in an M_2 population grown in a glasshouse under natural daylength conditions. The mutant line was bred by single plant selection for three generations before backcrossing to cultivar Solara. All *pcd1* material in this study derived from bulked *pcd1* segregates in the F_4 and F_5 generations of this cross.

For subsequent experiments, all seeds were sown either in a 1:1 mix of dolerite chips and vermiculite topped with potting soil (genetics and physiological experiments) or in vermiculite alone (biochemical experiments). Plants for all genetic experiments were grown in a heated glasshouse under natural daylight extended to 18 hr with light from a mixed fluorescent/incandescent source. The initial screen under monochromatic light and plants for chlorophyll (Chl) extractions were grown in growth cabinets at 20°C, using light sources as described by Weller et al. (1995). Light sources for the end-of-day far-red light (EOD-FR) experiment were identical to those described by Weller and Reid (1993). All other plants were grown in growth cabinets at 25°C. The red light (R) and FR sources used in other experiments and the green safety light used for manipulation of etiolated plants and during all biochemical experiments have been described by Nagatani et al. (1993). The fluence rates and spectra for all light sources were measured using a LiCor spectroradiometer (LiCor Corp., Lincoln, NE).

Protein Extraction and Immunoblotting

Crude protein extracts were prepared from dark-grown or R-treated seedlings and subjected to PAGE as described previously by Weller et al. (1995). The PHYA apoprotein was detected immunochemically (Weller et al., 1995), using the anti-pea phyA monoclonal antibody mAP5 (Nagatani et al., 1984). Protein was quantitated by the method of Bradford (1976), using Bio-Rad protein assay reagent and BSA as a standard.

Reagent Preparation

Biliverdin (BV) IXα was obtained from Porphyrin Products, Inc. (Logan, UT) and further purified by C18 reverse phase HPLC using a Shimadzu (Tokyo, Japan) LC10AS liquid chromatograph system and a Supelcosil LC-18 ODS column (4.6 × 25 cm; 5-mm particle diameter; Supelco Inc., Bellefonte, PA). The mobile phase consisted of ethanol–acetone–water–acetic acid (48:34:17:1 [v/v]; mobile phase A) with a flow rate of 1.5 mL/min, and the column eluate was monitored at 380 nm. HPLC-purified BV IXα was concentrated by diluting four times with 0.1% (v/v) trifluoro-acetic acid (TFA) and applying to a C18 Bond Elut column (1 mL; Varian, Harbor City, CA). After washing with 0.1% TFA, BV IXα was eluted with acetonitrile/0.1% TFA (3:2 [v/v]) and dried in vacuo. For BV feeding experiments to apical segments, BV (a mixture of isomers) was obtained from Sigma.

Phycocyanobilin (PCB) was purified from *Spirulina platensis* as described previously by Terry et al. (1993a). The sample contained >90% 3(*E*)-PCB when analyzed by HPLC. 3(*E*)-phytochromobilin (3[*E*]-PΦB) was a gift from J.C. Lagarias (University of California, Davis, CA). 3(*Z*)-PΦB was purified by HPLC after conversion of BV IXα to PΦB by isolated pea etioplasts (see below; Terry et al., 1995). HPLC conditions were identical to those for BV IXα purification, except that the mobile phase was changed to ethanol–acetone–100 mM formic acid (25:65:10 [v/v]; mobile phase B) to increase the resolution between BV IXα and 3(*Z*)-PΦB (Terry et al., 1995). All bilins were prepared as 1 mM stock solutions in DMSO, using the following molar absorption coefficients: 66,200 M cm^{-1} at 377 nm for BV IXα (McDonagh and Palma, 1980), 47,900 M cm^{-1} at 374 nm for 3(*E*)-PCB (Cole et al., 1967), and 64,570 M cm^{-1} at 386 nm and 38,020 M cm^{-1} at 382 nm for 3(*E*)-PΦB and 3(*Z*)-PΦB, respectively (Weller and Gossauer, 1980). Absorption spectrophotometry of bilin and heme samples was performed using a spectrophotometer (model U-3410; Hitachi, Tokyo, Japan).

A stock solution of heme was prepared by dissolving hemin chloride (Sigma) in 0.1 M NaOH and adjusting to pH 7.7 with 1 N HCl. The final heme concentration was 1 mM.

Unless otherwise stated, all chemicals and reagents were purchased from Sigma, Kanto Chemical Co. (Tokyo, Japan), and Nakalai Tesque (Kyoto, Japan).

Phytochrome Assembly in Vivo

For the experiments involving feeding of BV to floating shoot tips, 40 to 50 apical segments (2 cm long) from 5-day-old seedlings (~3 g fresh weight) were harvested in green safe-light under buffer (15 mM Hepes, NaOH, pH 7.4) and transferred to Petri dishes containing 300 μM of BV IXα in the same buffer. The apical segments were then cut into smaller segments, 3 to 4 mm in length, and floated with gentle shaking for 6 hr in darkness. After floating, the tissue was rinsed several times, blotted dry, weighed, frozen in liquid nitrogen, and stored at −80°C until phytochrome extraction.

Phytochrome Extraction and Assembly in Vitro

For each extraction, 40 to 50 apical segments (2 cm long) from 5-day-old etiolated seedlings (~3 g fresh weight) were harvested and homogenized in liquid nitrogen. The homogenate was suspended in a 3:1 (v/w) volume of extraction buffer (50 mM Tris, 100 mM ammonium sulfate, 25% [v/v] ethylene glycol, 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM diethyldithiocarbamate, 142 mM β-mercaptoethanol, 4 mM cysteine, 2 μg/mL leupeptin, 2 μg/mL aprotinin, 1 μg/mL pepstatin, adjusted with HCl to pH 8.3 at 5°C) and extracted with gentle stirring for 30 min at 4°C in the presence or absence of 3 μM PCB. After centrifugation for 30 min at 200,000*g*, saturated ammonium sulfate (0.725:1 [v/v]) was added to the supernatant, and the extract was gently stirred for an additional 30 min. The ammonium sulfate pellet was collected by centrifugation (30 min at 30,000*g*) and resuspended in 1 mL of TEGE buffer (25 mM Tris, 2 mM EDTA, 25% ethylene glycol [v/v], 2 mM PMSF, 1 mM DTT, 2 μg/mL leupeptin, adjusted with HCl to pH 7.8 at 5°C). The extract was then clarified by centrifugation (15 min at 200,000*g*) before spectrophotometric assay. Extracts were maintained at or below 4°C throughout the extraction. For assembly experiments with extracted apophyto-

chrome samples, the bilins were added to the clarified extract and incubated on ice for 30 min.

Spectrophotometric Assay for Phytochrome

All spectrophotometric determinations were made using a recording difference spectrophotometer (model 3410; Hitachi). Standard samples for in vivo spectrophotometric phytochrome determinations were prepared as described previously by Weller et al. (1995). All samples and extracts were kept at 4°C during measurements. Actinic beams for phytochrome photoconversion were obtained by filtering light from a xenon lamp (150 W; Ushio Inc., Tokyo, Japan) through a 658- or a 748-nm interference filter (Vacuum Optics Corp., Tokyo, Japan). Phytochrome was photoconverted by exposure to 60 sec of R and 90 sec of FR (in vivo determinations) or 180 sec of R and 300 sec of FR (in vitro), irradiations sufficient to induce essentially full photoconversion, as determined by kinetic measurements.

Assays for PΦB Synthesis

PΦB synthesis from heme and BV IXα was assayed in isolated pea etioplasts essentially as described previously for PΦB synthase assays in oat etioplasts (Terry et al., 1995). Etioplasts were isolated by differential centrifugation from 8-day-old dark-grown seedlings as described by Terry and Lagarias (1991) with the following modifications. PVP (0.5% [w/v]) was added to the homogenization medium, and the final crude plastid pellet was washed once with assay buffer stock (20 mM Tes, 10 mM Hepes, NaOH, pH 7.7, containing 500 mM sorbitol) before use.

PΦB synthesis assays were performed in 20 mM Tes, 10 mM Hepes, NaOH buffer, pH 7.7, containing 500 mM sorbitol, 1 mM PMSF, 0.5 mM DTT, 2 μM leupeptin, 3000 units/mL catalase, and an NADPH regenerating system (1.2 mM $NADP^+$, 10 mM glucose 6-phosphate, 1.5 units/mL glucose 6-phosphate dehydrogenase). The reaction was initiated by the addition of either heme (10 μM final concentration) or BV IXα (8 μM). For the assay with BV IXα, an argon treatment was used to deplete oxygen. The reaction volume was 0.5 mL, and reaction mixtures were incubated in the dark at 28°C with shaking. After a 3-hr incubation, samples were purified partially using a Bond Elut column (see above) as previously described by Terry et al. (1995). The elution volume was 1 mL. HPLC analysis of bilins was performed as described above using mobile phase A (Figures 8 and 10) or B (Figure 9).

Tetrapyrrole Quantitation

Heme Quantitation

Total noncovalently bound heme was extracted from the top 2 to 3 cm of 8-day-old dark-grown seedlings, essentially as described by Thomas and Weinstein (1990). Harvested tissue (5 g fresh weight) was homogenized in 20 mL of cold 90% (v/v) acetone containing 10 mM NH_4OH. After centrifugation at 4000*g* for 2 min, the pellet was washed twice more in the same volume. Noncovalently bound hemes were extracted twice with 10 mL of cold 80% (v/v) acetone containing 5% (v/v) HCl. The heme extracts were pooled, transferred to chloroform–butanol (2:1 [v/v]), and washed twice with water. The heme was then concentrated by application to a DEAE–Sepharose column (Bond Elut DEA, 3 mL; Varian) with an equal volume of 95% (v/v) ethanol. After washes with chloroform–butanol (2:1) and 95% ethanol, the heme was eluted in 2 mL of ethanol–acetic acid–water (70:17:13 [v/v]). The concentration was determined by absorption spectroscopy (see above), using the molar absorption coefficient of 144,000 $M^{-1}\,cm^{-1}$ at 398 nm calculated for air-oxidized protoheme in this solvent (Weinstein and Beale, 1983).

Chl Quantitation

Two 6-mm discs were punched from the sixth pair of stipules of 4-week-old plants and extracted in 2 mL of dimethylformamide for 24 hr at 4°C. Chl levels in the extracts were determined spectrophotometrically according to Innskeep and Bloom (1985).

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